Articles

2,4-Diamino-5-substituted-quinazolines as Inhibitors of a Human Dihydrofolate Reductase with a Site-Directed Mutation at Position 22 and of the Dihydrofolate Reductases from *Pneumocystis carinii* and *Toxoplasma gondii*

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2,4-Diaminoquinazoline antifolates with a lipophilic side chain at the 5-position, and in one case with a classical (*p*-aminobenzoyl)-L-glutamate side chain, were synthesized as potentially selective inhibitors of a site-directed mutant of human dihydrofolate reductase (DHFR) containing phenylalanine instead of leucine at position 22. This mutant enzyme is approximately 100-fold more resistant than native enzyme to the classical antifolate methotrexate (MTX), yet shows minimal cross resistance to the nonclassical antifolates piritrexim (PTX) and trimetrexate (TMQ). Although they were much less potent than trimetrexate and piritrexim, the lipophilic 5-substituted analogues were all found to bind approximately 10 times better to the mutant DHFR than to the wild-type enzyme. The potency of the analogue with a classical (*p*-aminobenzoyl)-L-glutamate side chain was similarly diminished in comparison with MTX, but the difference in its binding affinity to the two DHFR species was only 5-fold. Thus, by making subtle structural changes in the antifolate molecule, it may be possible to attack resistance due to mutational alterations in the active site of the target enzyme. Also, to test the hypothesis that DHFR from *Pneumocystis carinii* and *Toxoplasma gondii* may have a less sterically restrictive active site than the enzyme from mammalian cells, inhibition assays using several of the lipophilic analogues in the series were carried out against the *P. carinii* and *T. gondii* reductases in comparison with the enzyme from rat liver. In contrast to their preferential binding to mutant versus wild-type human DHFR, binding of these analogues to the *P. carinii* and *T. gondii* enzymes was weaker than binding to rat enzyme. It thus appears that, if the active site of the DHFR from these parasites is less sterically restrictive than the active site of the mammalian enzyme, this difference cannot be successfully exploited by moving the side chain from the 6-position to the 5-position.

2,4-Diaminoquinazolines with a bulky hydrophobic group at the 5-position were synthesized and evaluated as inhibitors of the enzyme dihydrofolate reductase (DHFR) from various species a number of years ago by Hynes and co-workers.[1,2] General types of compounds studied in this early series were 5-arylthio, 5-(arylthio)-methyl, 5-(2-arylethenyl), and 5-(2-arylethyl) derivatives; specific aryl groups included phenyl, 4-chlorophenyl, 3,4-dichlorophenyl, and 2-naphthyl. Sulfoxides and sulfones of some of the 5-arylthio and 5-(arylthio)methyl derivatives were also described. Activity was measured against rat liver and *Streptococcus faecium* enzyme, and several compounds were found to be potent inhibitors, with the bacterial enzyme generally showing greater sensitivity than the mammalian enzyme. Subsequently, another group[3] reported a large series of inhibitors featuring mainly nonaromatic groups at the 5-position, but also including a 5-benzyloxy and 5-(2-phenylethyl) derivative. Enzyme inhibition assays were carried out with these compounds against DHFR from several bacterial species and bovine liver, and extensive structure–activity analyses were performed with a view to correlating enzyme inhibition and growth inhibition. The structure–activity relationships were found to be complex, but, taken as a whole, activity against both bacterial and mammalian enzymes was unexceptional.

Our interest in 5-substituted 2,4-diaminoquinazolines was sparked by a recent molecular modeling study in which the three-dimensional features of the active site in wild-type human DHFR and a site-directed mutant enzyme with phenylalanine (Phe) in place of leucine (Leu) at position 22 were compared.[4] Interest in this particular mutation derives from the fact that position 22 is believed to be a "hot spot" for mutations in the *dhfr* gene. Although a natural $Leu^{22} \rightarrow Phe$ mutation in human DHFR remains to be found, such a mutation has been observed in two different Chinese hamster ovary cell lines selected for resistance to the classical antifolate methotrexate (MTX, **1**; Chart 1) in two independent laboratories.[5,6] In addition to the $Leu^{22} \rightarrow Phe$ mutation, a $Leu^{22} \rightarrow Arg$ mutation has been reported in a murine transformed fibroblast cell line, 3T6-R400.[7] The $Leu^{22} \rightarrow Phe$ mutant is able to reduce dihydrofolate normally, but the binding of MTX is decreased 100-fold

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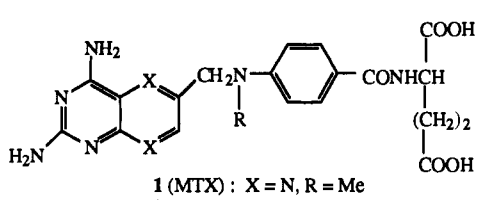
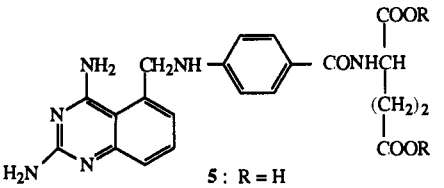
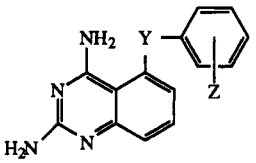
Chart 1



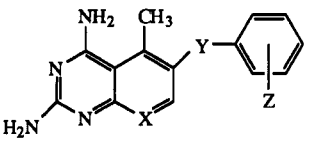
1 (MTX) : X = N, R = Me
2 (AMT) : X = N, R = H
3 : X = CH, R = H



5 : R = H
15 : R = Me



6 : Y = CH_2NH, Z = 2',5'-$(OMe)_2$
7 : Y = CH_2NH, Z = 3',4',5'-$(OMe)_3$
8 : Y = $CH_2N(Me)$, Z = 2',5'-$(OMe)_2$
9 : Y = $CH_2N(Me)$, Z = 3',4',5'-$(OMe)_3$
10 : Y = CH_2CH_2, Z = 2',5'-$(OMe)_2$
11 : Y = CH_2CH_2, Z = 3',4',5'-$(OMe)_3$



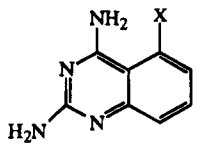
4 (PTX) : X = N, Y = CH_2, Z = 2',5'-$(OMe)_2$
12 (TMQ) : X = CH, Y = CH_2NH, Z = 3',4',5'-$(OMe)_3$

as a result of a sterically unfavorable interaction between the phenyl ring of the MTX and the Phe residue of the enzyme. Not surprisingly, the binding of aminopterin (**2**) is decreased by a similar amount. In contrast, the binding of 5,8-dideazaAMT (**3**) was decreased only 11-fold and that of a lipophilic DHFR inhibitor, piritrexim (PTX, **4**), remained essentially unchanged. An intriguing prediction of this molecular modeling study was that binding to the mutant enzyme would be enhanced if the (*p*-aminobenzoyl)glutamate moiety in MTX or other classical antifolates were moved to the 5-position. To enable this prediction to be tested, we synthesized the previously unknown compound *N*-[4-[[(2,4-diaminoquinazolin-5-yl)methyl]amino]benzoyl]-L-glutamic acid (**5**). In addition we prepared a series of 5-substituted 2,4-diaminoquinazolines (**6**–**11**) that lack a (*p*-aminobenzoyl)glutamate moiety and can thus be viewed as analogues of PTX and another nonclassical antifolate, the quinazoline trimetrexate (TMQ, **12**). This would enable the effect of moving the side chain from the 6- to the 5-position in nonclassical as well as classical antifolates to be compared and would constitute a rare, if not the first, example of a "designer antifolate" targeted to a site-directed mutant of human DHFR. It may be noted that in none of the previous studies on 2,4-diamino-5-substituted quinazoline DHFR inhibitors[1-3] did the bridge between the two aryl moieties contain nitrogen.

A second purpose in making the lipophilic analogues **6**–**11** was to determine whether they were selective inhibitors of DFHR from *Pneumocystis carinii* and *Toxoplasma gondii*, two opportunistic pathogens that produce significant morbidity and mortality in patients with AIDS and other immunodeficiency disorders.[8] PTX and TMQ have been used to treat AIDS patients who are infected with these organisms and are either refractory to, or cannot tolerate, sulfamethoxazole, pentamidine, or other standard antiparasitic drugs.[9-12] However, because PTX and TMQ bind much better to human DHFR than to the *P. carinii* or *T. gondii* enzyme, leucovorin has to be co-administered in order to prevent potentially life-threatening hematotoxicity. The basis of this selective "rescue" strategy is that *P. carinii* and *T. gondii*, unlike mammalian cells, lack the ability to take up reduced folates by carrier-mediated active transport and are therefore impermeant to leucovorin.[13] A recent molecular dynamics analysis of the binding of MTX to *P. carinii* DHFR suggests that the active site is somewhat larger in the *P. carinii* enzyme than in DHFR from other species and may therefore be less subject to unfavorable van der Waals interactions.[14] If this theoretical prediction is correct, one might expect 2,4-diamino-5-substituted-quinazolines to perhaps bind better to *P. carinii* DHFR than to mammalian DHFR. Assuming that the drug were efficiently taken up by the intact organism, the need for leucovorin rescue might be obviated.

Chemistry

Of the various routes available for the synthesis of 2,4-diamino-5-substituted-quinazolines, the most attractive were (i) reductive coupling between a substituted arylamine and 2,4-diaminoquinazoline-5-carbonitrile (**13**), and (ii) Pd(0)-mediated Heck coupling between 2,4-diamino-5-iodoquinazoline (**14**) and an arylacetylene, with subsequent reduction of the ethynyl bridge. Iodide **14** was made according to the method of Hynes and co-workers,[15] starting from commercially available 2,6-difluorobenzonitrile, whereas nitrile **13** was made by a new method involving replacement of the iodo group in **14** by heating with cuprous cyanide and sodium cyanide in DMF at 120 °C. It may be noted that **13** was made earlier by Hynes and co-workers[16] from 3-fluorophthalonitrile, which was prepared from 2-amino-6-fluorobenzonitrile via a Sandmeyer reaction. The latter, in turn, was made from 2,6-difluorobenzonitrile by heating it with dry gaseous ammonia in DMSO. An attractive feature of our approach was that it avoided the need to prepare 3-fluorophthalonitrile, i.e., **14** was not only an end product in itself but was also an intermediate to **13**.



13 : X = CN
14 : X = I
16 : X = $-C{\equiv}CC_6H_5$
17 : X = $-CH{=}CHC_6H_5$
18 : X = $-CH_2CH_2C_6H_5$
19 : X = $-C{\equiv}CC_6H_3$(2',5'-$OMe)_2$
20 : X = $-C{\equiv}CC_6H_2$(3',4',5'-$OMe)_3$

Reductive condensation of **13** with an equimolar amount of 3,4,5-trimethoxyaniline in glacial acetic acid

in the presence of Raney Ni and hydrogen for 4 h gave a 37% yield of **7** after silica gel chromatography and recrystallization. The same reaction with the more sterically hindered 2,5-dimethoxyaniline gave **6**, but in only 27% yield despite the use of excess amine. Elslager and co-workers[17] reported a 26% yield for the reductive coupling of 3,4,5-trimethoxyaniline and 2,4-diamino-quinazoline-6-carbonitrile. Thus, while the reaction may be sensitive to *ortho* substitution in the arylamine, it does not appear to be influenced by whether the nitrile group is at the 6-position or the sterically more hindered 5-position of the quinazoline. The effect of *ortho* substitution was also evident in the N-methylation reaction with formaldehyde and sodium cyanoborohydride, which after silica gel chromatography and recrystallization afforded a 69% yield of **9** but only a 31% yield of **8**, along with 13% recovery of unchanged **6**. Reductive coupling of **13** and dimethyl *N*-(4-aminobenzoyl)-L-glutamate followed by alkaline hydrolysis of the esters afforded **15** and **5**, respectively. Unfortunately the yield in the coupling step was only 10–15%, presumably reflecting a combination steric hindrance and deactivation of the aromatic amino group by the electron withdrawing CONH group.

The structures of **6–9** were confirmed by elemental analysis and on the basis of 500 MHz ^{1}H NMR spectra in DMSO-d_6, which showed some interesting features relating to the magnetic environment around the CH_2 protons at C^9 and the CH_3 protons at N^{10}. The C^9 protons in the N^{10}-unsubstituted compounds **6** and **7** gave doublets at δ 4.41 and 4.29, respectively, whereas the corresponding doublets in the spectra of the N^{10}-methyl derivatives **8** and **9** were at δ 4.22 and 4.47. Thus, the 2′-methoxy group produced a downfield displacement of the C^9 proton singlet when N^{10} was unsubstituted (compare **6** and **7**), but an upfield displacement when N^{10} was methylated (compare **8** and **9**). Furthermore, the effect of N^{10}-methylation on the chemical shift of the C^9 protons depended on whether a 2′-methoxy group was present or absent, with N^{10}-methylation causing a downfield shift of the C^9 proton signal in **8** relative to **6** and an upfield shift in **9** relative to **7**. It may also be noted that the chemical shift for the N^{10}-methyl protons themselves depended on the presence or absence of a 2′-methoxy group. Interestingly, in contrast to the effect of 2′-methoxy group on the chemical shift of the C^9 protons, the effect of 2′-methoxy substitution on the N^{10}-methyl signal was an upfield displacement (compare **8** and **9**). These subtle magnetic effects are likely to represent sterically determined adjustments in the dihedral angle about the C^9–N^{10} bond. The resulting changes in spatial orientation of the phenyl and quinazoline moieties should in turn affect enzyme binding.

The 500 MHz ^{1}H NMR spectrum of **5** showed the expected bridge and side-chain features and in addition displayed three sets of multiplets for the aromatic protons of the quinazoline. The most downfield quinazoline signal (δ 7.44) was assigned to the C^8 proton on the basis of its proximity to the electron-withdrawing N^1. The most upfield signal (δ 7.07) was tentatively assigned to C^6 proton on the basis that the 9-CH_2 group would be expected to produce second-order coupling as well as long range shielding by the unshared electrons of the 10-NH group or the π-electons of the phenyl ring. By elimination, therefore, the signal at δ 7.21 was assigned to the C^7 proton. Similar reasoning was used to assign chemical shifts to the quinazoline protons of the other 5-(arylamino)methyl derivatives **6–9**.

We had some initial concerns about whether the Heck reaction would work with **14**. These concerns related to (i) the relatively crowded environment at C^5 and (ii) the possibility that the 4-amino group might participate in the Pd(0)-mediated reaction to produce a fused five-membered ring.[18] Accordingly, we carried out a model sequence using commercially available phenylacetylene. Heating an equimolar mixture of **14** and phenylacetylene in the presence of $PdCl_2$, triphenylphosphine, and CuI for 5 h in refluxing MeCN produced 2,4-diamino-5-(2-phenylethynyl)quinazoline (**16**) in 21% yield after silica gel chromatography and recrystallization. Catalytic hydrogenation of **16** in the presence of 10% Pd–C for 7 h led to the *cis* olefin **17**, and a longer reaction time of 20 h afforded **18**. However, complete reduction proved rather difficult and tended to give mixtures of **17** and **18** that had to be separated by chromatography. The large amount of Pd–C catalyst which has to be used in this reduction may result in poor product recovery once the bridge is saturated. An effort to alleviate this problem was made by converting **16** to a trifluoroacetate salt and carrying out the hydrogenation in DMF.[19] Despite these measures, the yield of the saturated product **18** after chromatography and recrystallization was only 37%, whereas it had been possible to isolate a 58% yield of the olefin **17**.

The structures of compounds **16–18** were confirmed by their 500 MHz ^{1}H NMR spectra in DMSO-d_6 solution. As expected, the spectrum of **16** contained only aromatic proton signals in addition to the two broad peaks for the 2- and 4-amino groups. The quinazoline protons at the 7- and 8-positions yielded the predicted pair of closely spaced doublets at δ 7.24 and 7.27. In the spectrum of **17** a pair of doublets at δ 6.93 (J = 12 Hz) and δ 7.10 (J = 12 Hz) established that the double bond has the expected *cis* configuration. On the basis of the probable shielding effect of the 4-amino group, we tentatively assign the δ 6.93 signal to the C^9 proton and the δ 7.10 signal to the C^{10} proton. In the spectrum of **18** these signals were replaced by a pair of triplets at δ 3.43 and 2.86, provisionally assigned to the C^9 and C^{10} protons, respectively.

Since the primary purpose of the foregoing experiments using phenylacetylene had been to determine whether Heck reactions would work with **14**, we did not spend any more time in optimizing the reduction of **16** to **18** but moved instead to the synthesis of the target compounds **10** and **11** via the acetylenes **19** and **20**. Condensation of **14** with (3,4,5-trimethoxyphenyl)acetylene[20] in the presence of $PdCl_2$, triphenylphosphine, and CuI was performed the same way as with phenylacetylene except that the reaction time was extended to 18 h. Despite this longer reaction time, the yield of **19** after chromatography and recrystallization was still only 30%. On the other hand when the reaction was done with (2,5-dimethoxyphenyl)acetylene,[21] the yield of **20** was 42%, suggesting that the *o*-methoxy group may increase the susceptibility of the triple bond to take part in Pd(0)-mediated coupling. Hydrogenation of the trifluoroacetate salt of **19** and **20** in DMF solution in the presence of 10% Pd–C for 1 h afforded the saturated

Table 1. Inhibition of Wild-Type and Leu_{22}→Phe Mutant Human Dihydrofolate Reductase by a Classical 2,4-Diaminoquinazoline Antifolate with the Side Chain at the 5-Position

compound[a]	K_i (nM) wild-type	mutant	mutant/wild-type
1 (MTX)	0.0012	0.11	88
2 (AMT)	0.0018	0.21	120
3 (5,8-dideazaAMT)	0.00089	0.010	11
5	0.54 ± 0.013	2.7 ± 0.1	5.0

[a] Inhibition constants K_i were determined according to ref 22.

products **10** (71%) and **11** (34%). The higher yield of **10** in this reduction was unexpected and may simply have been due to better recovery of the product from the hydrogenation catalyst.

The 500 MHz ^1H NMR spectrum of **11**, taken in DMSO-d_6 solution, showed a pair of triplets at δ 2.79 and 3.42. The corresponding triplets in the spectrum of **10** were at δ 2.80 and 3.17. Thus the triplet with the higher chemical shift was assigned to the C^9 protons, which in the case of **10** were shielded by the *o*-methoxy group. This was also consistent with our peak assignment to the C^9 and C^{10} protons in **18** (see above).

Enzyme Inhibition

Inhibition constants K_i of compounds **5**–**11** against wild-type human DHFR and the Leu^{22}→ Phe mutant were determined as previously described for the binding of MTX to site-directed Phe^{31}→Ser and Phe^{34}→Ser mutants of human DFHR.[22] The results are summarized in Tables 1 and 2, which also include reference data for MTX, AMT, 5,8-dideazaAMT (**3**), PTX, and TMQ. As indicated, in Table 1, the K_i values of MTX and AMT were 0.0012 and 0.0018 nM against the wild-type enzyme, but 0.11 and 0.21 nM against the mutant enzyme, corresponding to roughly a 100-fold increase in K_i in both instances. In contrast, the K_i values of **3** against the wild-type enzyme and mutant enzyme were 0.000 89 and 0.010 nM. Thus, in addition to being a slightly better inhibitor of the wild-type enzyme than AMT, the quinazoline analogue showed a decrease in binding to the mutant enzyme of only 11-fold, i.e., there was only partial cross-resistance. These results suggest that 2,4-diaminoquinazoline and 2,4-diaminopteridine classical antifolates may bind to DHFR in different conformations. Direct evidence addressing this possibility, e.g., via X-ray crystallographic analysis, would be of considerable interest.

The 5-substituted analogue **5** was a weaker inhibitor of both wild-type DHFR and the Leu^{22}→Phe than AMT, with K_i values of 0.54 and 2.7 nM, respectively; however the difference in binding affinity for the two enzymes was only 5-fold. This provided the first experimental evidence that moving the (*p*-aminobenzoyl)-L-glutamate side chain in a classical inhibitor from C^6 to C^5 can at least partly offset the unfavorable steric and van der Waals interactions created by the Leu^{22}→Phe mutation. Though the potency of **5** was lower than that of the 6-substituted analogues, the fact that the decrease in binding to the mutant DHFR was relatively minor suggests that the synthesis of additional compounds of this type may be worthwhile, especially if potency can also be improved by making other structural changes. However, it is important to keep in mind that *in vivo* activity against a tumor cell with a Leu^{22}→Phe mutation in its DHFR would require that moving the side chain from C^6 to C^5 still allows efficient cellular transport and retention of the drug.

As indicated in Table 2, the binding of the glutamate-lacking compounds **6**–**11** and **16**–**19** to the two species of DHFR yielded a number of intriguing structure–activity correlations. Considering first the nature of the bridge, it is clear that rigidly linear C≡C derivatives bind much less well than the corresponding CH_2CH_2 analogues to both the wild-type enzyme and the Leu^{22}→Phe mutant (cf. **16** versus **18**; **19** versus **10**). However, a *cis*-CH=CH bridge is more favorable than a CH_2CH_2 bridge (cf. **17** versus **18**), suggesting that coplanarity between the parts of the molecule may be advantageous provided that the correct spatial orientation is maintained. In fact, **17** appears to be a better inhibitor of both the wild-type and the mutant enzyme than PTX and TMQ despite the fact that it lacks methoxy groups. While it would obviously have been of interest to examine the role of double-bond geometry, the *trans* analogue of **17** was not available for comparison. It also remains to be determined whether the favorable properties of a *cis*-CH=CH bridge apply to substituted phenyl derivatives. Another conclusion that can be drawn from the data is that a CH_2NH or $CH_2N(Me)$ bridge is likewise unfavorable in comparison with a CH_2CH_2 bridge (cf. **6** and **8** versus **10**; **7** and **9** versus **11**). Perhaps most striking, however, is that the 5-substituted-2,4-diaminoquinazolines, unlike the classical antifolates MTX, AMT, and 5,8-dideazaAMT (cf. Table 1) and the nonclassical antifolates PTX and TMQ, are better inhibitors of the mutant enzyme than of the wild-type enzyme. This was most clearly seen by examining the ratios of K_i values, which were all <1.0. For PTX and TMQ, these ratios were 1.2 and 6.4, whereas for MTX, AMT, and 5,8-dideazaAMT they were 88, 120, and 11. The most selective member of the series was **11**, whose K_i values of 4.3 and 72 nM produced a ratio of 0.06. Thus one may say that there is approximately a 20-fold improvement in binding selectivity relative to PTX (i.e., 1.2/0.06), a 100-fold improvement relative to TMQ (i.e., 6.4/0.06), and an even greater improvement relative to MTX, AMT, and 5,8-dideazaAMT. Moreover, it is worth noting that four of the analogues were better inhibitors of the mutant enzyme than PTX and TMQ were of the wild-type enzyme.

The fact that the MTX- and AMT-resistant Leu^{22}→Phe mutant of human DHFR is still somewhat resistant to **5** but is more sensitive than the wild-type enzyme to nonclassical analogues in which the side chain is moved from the 6- to the 5-position suggests that the mode of binding of these two types of 5-substituted analogues may be different. This may reflect a strong contribution by the (*p*-aminobenzoyl)-L-glutamate α-COOH group in **5**, which may force the molecule to bind in a spatial orientation different from that of compounds with only an aromatic ring as the side chain. A potentially advantageous feature of nonclassical analogues such as **9**–**11**, **17**, and **18** relative to **5** is that, by analogy with the uptake of PTX and TMQ, the mode of entry of these compounds into cells is likely to be by diffusion as opposed to active transport.

The ability of compounds **7**–**11** and **16**–**19** to inhibit

Table 2. Inhibition of Wild-Type and Leu_{22}→Phe Mutant Human Dihydrofolate Reductase by 2,4-Diaminoquinazoline Nonclassical Antifolates with the Side Chain at the 5-Position

compound[a]	bridge	K_i (nM ± SD) wild-type	K_i (nM ± SD) mutant	mutant/wild-type
unsubstituted				
16	C≡C	>500	96.3 ± 33	<0.19
17	CH=CH (*cis*)	5.4 ± 0.6	1.8 ± 0.2	0.33
18	CH_2CH_2	23 ± 2.3	2.9 ± 0.1	0.13
2′,5-dimethoxy				
6	CH_2NH	200 ± 26	33 ± 1.9	0.17
8	$CH_2N(Me)$	>500	250 ± 12	<0.50
10	CH_2CH_2	41 ± 5.0	4.2 ± 0.4	0.10
19	C≡C	>500	140 ± 31	<0.28
3′,4′,5′-trimethoxy				
7	CH_2NH	>500	310 ± 23	<0.62
9	$CH_2N(Me)$	>500	49 ± 4.7	<0.10
11	CH_2CH_2	72 ± 5.3	4.3 ± 0.2	0.06
reference compounds				
4 (PTX)	CH_2	33	38	1.2
12 (TMQ)	CH_2NH	13	83	6.4

[a] Data for PTX are from ref 4. For clarity, numbers are rounded off to two significant figures. Inhibition constants K_i were determined according to ref 21.

Table 3. Inhibition of *Pneumocystis carinii*, *Toxoplasma gondii*, and Rat Liver Dihydrofolate Reductase by Nonclassical 2,4-Diaminoquinazolines with the Side Chain at the 5-Position

compound[a]	rat liver (IC_{50}, μM)	*P. carinii* IC_{50} (μM)[b]	*P. carinii* selectivity[c]	*T. gondii* IC_{50} (μM)[b]	*T. gondii* selectivity
unsubstituted					
16	>30	>30	*d*	>30	*d*
17	1.5	10	0.15	1.2	1.3
18	0.51	3.2	0.16	0.37	1.4
2′,5′-dimethoxy					
6	>5	>5	*d*	>5	*d*
8	84	>40	*d*	>40	*d*
10	4.1	19	0.21	5.9	0.69
19	>18	>18	*d*	>18	*d*
3′,4′,5′-trimethoxy					
7	>20	>20	*d*	>20	*d*
9	53	>20	*d*	>20	*d*
11	3.7	14	0.26	0.93	4.1
reference compounds					
PTX (**4**)	0.0015	0.031	0.048	0.017	0.088
TMQ (**12**)	0.003	0.042	0.071	0.010	0.30
pyrimethamine	2.3	3.7	0.62	0.39	7.7
trimethoprim	130	12	11	2.7	48

[a] Data shown for TMQ, PTX, pyrimethamine, and trimethoprim as reference compounds are adapted from ref 23. For clarity, numbers are rounded off to two significant figures. [b] For values only listed as being greater than a given concentration, the limited solubility limit of the compound prevented the IC_{50} from being reached. IC_{50} values were determined spectrophotometrically according to refs 24 and 25. [c] Selectivity is defined as the ratio IC_{50}(rat)/IC_{50}(*P. carinii*) or IC_{50}(rat)/IC_{50}(*T. gondii*). [d] Not evaluable because of low solubility.

DHFR from *P. carinii*, *T. gondii*, and normal rat liver was also assessed, with the aim of determining whether there might be selective binding to the former two enzymes. Unfortunately, however, only four of them (**10**, **11**, **17**, and **18**) were soluble enough in the final assay mixture to allow the IC_{50} to be reached. All four were 5−10-fold better inhibitors of rat liver DHFR than of *P. carinii* DHFR and thus lacked the selectivity we had hoped to find if the hypothesis were correct that the active site of *P. carinii* DHFR is less sterically restrictive than that of other species.[14] The best inhibitor of the *P. carinii* enzyme was **18**, with an IC_{50} of 3.2 μM. Interestingly, the corresponding unsubstituted compound with a CH=CH rather than CH_2CH_2 bridge was several times less active against the rat enzyme, whereas the opposite had been noted against the human enzyme (Table 1). However, this may simply reflect differences in the assay methodology. The reference compound whose profile **18** resembled most closely in its interaction with the *P. carinii* enzyme was pyrimethamine, with an IC_{50} of 3.7 μM and a selectivity ratio of 0.62. The best inhibitor of *T. gondii* DHFR was likewise **18**, with an IC_{50} of 0.37 μM. Although this value was essentially the same as the IC_{50} of pyrimethamine, the selectivity ratio of **18** was only 1.4, whereas that of pyrimethamine was 7.7. Contrary to expectations, dimethoxy as well as trimethoxy substitution of the 5-phenyl group (cf. **10** and **11** versus **18**) produced a decrease in binding to all three enzyme species. Moreover the same effect occurred with the wild-type and mutant human enzymes. It thus appears that the introduction of hydrophobic methoxy substituents in the 5-aralkyl group improves neither potency nor selectivity where *P. carinii* and *T. gondii* DHFR inhibition is concerned. If the active site of the enzyme from these parasites is less sterically restrictive than the active site of the mammalian enzyme, it appears that this difference cannot be successfully exploited by moving the side chain from the 6-position to the 5-position.

In summary, a pilot group of nonclassical antifolates with the hydrophobic side chain at the 5-position instead of the 6-position were found to bind better to a site-directed mutant (Leu^{22}→ Phe) of human DHFR than

they do to the wild-type human enzyme, suggesting that this is potentially a useful way to overcome MTX resistance due to this particular alteration of the active site, provided that greater potency can be achieved as well as selectivity for the mutant. The same strategy, appropriately tailored to other active site mutations in the enzyme target, may also have applications in the broader context of antifolate selectivity and resistance. Moving the hydrophobic side chain to the 5-position unfortunately failed to produce selectivity against *P. carinii* or *T. gondii* DHFR relative to mammalian DHFR. Nonetheless, given the urgent need for safe and effective drugs for the management of PCP and toxoplasmosis in patients with AIDS, and the likelihood that resistance will become as problematic in the treatment of recurrent PCP infections with antifolates as it is in cancer chemotherapy, the design of different types of fused 2,4-diaminopyrimidine derivatives combining the high potency of TMQ and PTX with the high selectivity of pyrimethamine and trimethoprim remains a worthwhile goal.

Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer; only peaks with wavenumbers greater than 1500 cm^{-1} are reported. High-resolution ^1H NMR spectra were recorded on a Varian Model VXR500 instrument, using Me_4Si as the reference. TLC analyses were done on Whatman MK6F silica gel plates, with spots being visualized under 254-nm illumination. Unless otherwise specified, the developing solvent for TLC consisted of 100:10:1 $CHCl_3$–MeOH–28% NH_4OH (solvent A). Column chromatography was on Baker 7024 flash silica gel (40 μm particle size) with 9:1 CH_2Cl_2–MeOH (solvent B), 10:1 CH_2Cl_2–MeOH (solvent C), or 100:10:1 CH_2Cl_2–MeOH–28% NH_4OH (solvent D) as the eluent. Solvents for moisture sensitive reactions were purchased from Aldrich in Sure/Seal bottles. Melting points were determined in Pyrex capillary tubes using a Mel-Temp Apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. Microanalyses were performed by QTI Laboratories, Whitehouse, NJ.

2,4-Diaminoquinazoline-5-carbonitrile (13). A mixture of NaCN (769 mg, 15.7 mmol) and CuCN (1.4 g, 15.7 mmol) in dry DMF (30 mL) was heated to 120 °C under an argon atmosphere, and 2,4-diamino-5-iodoquinazoline (**14**) (4.5 g, 15.7 mmol)[15] was added in small portions to the resulting clear solution. After 4 h at 120 °C, the reaction mixture was cooled and concentrated to dryness with the aid of a rotary evaporator connected to a vacuum pump. The orange residue was stirred overnight with 1 M 1,2-ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) (200 mL) adjusted dropwise to pH 8 with 28% NH_4OH. The solid was filtered and treated twice more with 1 M EDTA (200 mL) for 2 h and then filtered and washed well with H_2O. The solid was digested with boiling MeOH (300 mL) and filtered. The undissolved red solid was again digested with boiling MeOH (100 mL) and filtered, and the combined filtrates were concentrated by rotary evaporation to obtain an orange solid. Chromatography on silica gel with solvent C as the eluent gave the desired product as an off-white powder (1.34 g, 46% yield), identical in all respects to the previously reported sample: mp 234–236 °C (lit.[16] mp 231–232 °C); R_f 0.34 (silica gel, solvent A).

Dimethyl *N*-[4-[[(2,4-Diaminoquinazolin-5-yl)methyl]amino]benzoyl]-L-glutamate (15). A mixture of nitrile **13** (0.5 g, 2.70 mmol), dimethyl *N*-(4-aminobenzoyl)-L-glutamate (1.18 g, 4.05 mmol), NaOAc (30 mg), and Raney Ni (50% in H_2O, 200 mg) in glacial AcOH (20 mL) was hydrogenated at 50 lb/in.^{2} in a Parr apparatus for 6 h. The reaction mixture was filtered through Celite, the pad was washed with glacial AcOH, and the filtrate was concentrated by rotary evaporation. The residue was taken up in H_2O, and the solution cooled in an ice bath while adjusting the pH to neutrality with 28% NH_4OH. The precipitated solid was collected, washed with H_2O, and purified by silica gel column chromatography with solvent D as the eluent. Fractions giving a single TLC spot (R_f 0.15, silica gel, solvent A) were pooled and evaporated to a white solid, which was recrystallized from boiling EtOH to obtain white needles (135 mg, 11%); mp 168–171 °C dec (softening above 158 °C); IR (KBr) ν 3420 br, 2940, 1735, 1630, 1610, 1570, 1560, 1500 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.08 m, 2H, β-CH_2), 3.57 (s, 3H, γ-COOMe), 3.61 (s, 3H, α-COOMe), 4.40 (m, 1H, α-CH), 4.45 (m, 2H, 9-CH_2), 5.98 (s, 2H, 2-NH_2), 6.78–6.80 (m, 3H, 3'- and 5'-H, 10-NH), 6.99 (s, 2H, 4-NH_2), 7.03 (m, 1H, 6-H), 7.18 (m, 1H, 7-H), 7.40 (m, 1H, 8-H), 7.72 (d, 2H, 2'- and 6'-H), 8.37 (d, 1H, amide NH). Anal. ($C_{23}H_{26}N_6O_5 \cdot 0.8H_2O$) C, H, N.

***N*-[4-[[(2,4-Diaminoquinazolin-5-yl)methyl]amino]benzoyl]-L-glutamic Acid (5). A.** A solution of **15** (77 mg, 0.16 mmol) in MeOH (1.5 mL) was treated with 0.5 N NaOH (0.1 mL) and stirred at room temperature for 24 h. The pH was adjusted to 6 with 10% AcOH, the mixture was chilled for 2 h, and the pale cream-colored precipitate was filtered and dried in vacuo over P_2O_5 at 55 °C for 3 d. Preparative HPLC (C_{18} silica gel, 9% MeCN in 0.05 M NH_4OAc, 10 mL/min) and freeze-drying of collected eluates afforded **5** as a white solid (31 mg, 42%).

B. Solid $Ba(OH)_2 \cdot H_2O$ (83 mg, 0.44 mmol) was added to a suspension of **15** (100 mg, 0.22 mmol) in 50% MeOH–H_2O (4.4 mL), and the mixture was stirred overnight. A solution of NH_4HCO_3 (100 mg) in H_2O (20 mL) was then added, and after 20 min the solid was filtered (sintered-glass funnel) and washed with H_2O. The filtrate was acidified to pH 3 with AcOH, the precipitated solid was filtered, and the filtrate was freeze-dried; yield 30 mg. The original solid from the sintered-glass funnel was extracted again with 5% NH_4OH (2 × 50 mL), washed with H_2O (2 x 20 mL), and filtered. The combined filtrates were freeze-dried; yield 37 mg (total 67 mg, 71%). Ion-exchange on a DEAE-cellulose using NH_4HCO_3 (0.2 to 2.4 M) as the eluent, followed by freeze-drying, afforded a white solid indistinguishable (mp, IR, ^1H NMR) from the product in the preceding experiment; mp 205–209 °C dec; IR (KBr) ν 3420 br, 1640, 1600, 1570, 1500 cm-1; ^1H NMR (DMSO-d_6) δ 1.93–2.28 (m, 4H, CH_2CH_2), 4.30 (br s, 1H, α-CH), 4.4 (s, 2H, 9-CH_2), 6.25 (s, 2H, 2-NH_2), 6.74 (m, 1H, 10-NH), 6.79 (d, 2H, 3'- and 5'-H), 7.07 (m, 1H, 6-H), 7.17 (s, 2H, 4-NH_2), 7.21 (m, 1H, 7-H), 7.44 (m, 1H, 8-H), 7.69 (d, 1H, 2'- and 6'-H), 8.0 (br s, 1H, CONH). Addition of D_2O caused disappearance of the 2-NH_2, 4-NH_2, and CONH protons. Anal. ($C_{21}H_{22}N_6O_5 \cdot 0.1NH_3 \cdot 3.5H_2O$) C, H, N.

2,4-Diamino-5-[(2',5'-dimethoxyanilino)methyl]quinazoline (6). A slurry of Raney Ni (100 mg, 50% in H_2O) was added to a solution of **13** (500 mg, 2.70 mmol) and 2,5-dimethoxyaniline (600 mg, 3.92 mmol) in glacial AcOH (20 mL), and the mixture was hydrogenated in a Parr apparatus (30 lb/in.^{2} initial pressure) for 2 h. At this time the reaction mixture was treated with additional catalyst (100 mg, 50% in H_2O) and 2,5-dimethoxyaniline (200 mg, 1.31 mmol), and hydrogenation at 30 lb/in.^{2} pressure was resumed for 2 h. The mixture was filtered through a pad of Celite, which was washed with glacial AcOH. The filtrate was concentrated to 2 mL by rotary evaporation, diluted with 50 mL of H_2O, and neutralized dropwise with 28% NH_4OH. The precipitated solid was collected, stirred in cold 0.5 N NaOH (10 mL) for 5 min, filtered, washed with H_2O, and chromatographed on silica gel with solvent D as the eluent. Pooled TLC-homogeneous fractions with R_f 0.28 (silica gel, solvent A) were recrystallized from boiling EtOH–H_2O to obtain **6** as off-white needles (239 mg, 27% yield): mp 180–181 °C; IR (KBr) ν 3400 (br), 1640, 1605, 1560, 1510 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.65 (s, 3H, 5'-OMe), 3.69 (s, 3H, 2'-OMe), 4.41 (d, 2H, CH_2N), 5.05 (m, 1H, NH), 6.00 (br s, 2H, NH_2), 6.25 (d, 1H, 3'- or 4'-H), 6.38 (d, 1H, 3'- or 4'-H), 6.76 (d, 1H, 6'-H), 7.01 (d, 1H, 6-H), 7.16 (br m, 3H, NH_2 and 7-H, overlapping), 7.39 (m, 1H, 8-H). Anal. ($C_{17}H_{19}N_5O_2 \cdot 1.3H_2O$) C, H, N.

2,4-Diamino-5-[(3',4',5'-trimethoxyanilino)methyl]quinazoline (7). A slurry of Raney Ni (100 mg, 50% in H_2O) was added to a solution of **13** (500 mg, 2.7 mmol) and 3,4,5-trimethoxyaniline (494 mg, 2.7 mmol) in glacial AcOH (20 mL),

and the mixture was hydrogenated in a Parr apparatus (35 lb/in.^{2} initial pressure) for 3 h. After filtration through a Celite pad and washing with glacial AcOH, the filtrate was concentrated to 2 mL by rotary evaporation and neutralized dropwise with 28% NH_4OH. The precipitate was collected, washed with 5% NH_4OH (2 × 10 mL) and H_2O (50 mL), and purified by chromatography on silica gel with solvent C as the eluent. The desired product (TLC: R_f 0.20; silica gel, solvent A) was obtained as a white solid, and yielded needles on recrystallization from 1:4 CH_2Cl_2–MeOH: yield 0.36 g, 37%; mp 224 °C dec; IR (KBr) ν 3460, 3340, 3100, 2920, 1650, 1630, 1605, 1575, 1550, 1510 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.54 (s, 3H, 4′-OMe), 3.70 (s, 6H, 3′- and 5′-OMe), 4.29 (d, 2H, CH_2N), 5.95 (s, 2H, 2′- and 6′-H), 6.00 (br s, 1H, bridge NH), 6.15 (s, 2H, NH_2), 7.02 (m, 1H, 6-H), 7.18 (m, 1H, 7-H), 7.35 (br m, 2H, NH_2), 7.40 (m, 1H, 8-H). Anal. ($C_{18}H_{21}N_5O_3$·0.2H_2O) C, H, N.

2,4-Diamino-5-[(*N*-methyl-2′,5′-dimethoxyanilino)-methyl]quinazoline (8). A stirred mixture of **6** (179 mg, 0.55 mmol) and 37% HCHO (0.55 mL, 0.55 mmol) in MeCN (15 mL) at room temperature was treated with 1 M $NaCNBH_3$ in THF (1.76 mL) followed by dropwise addition of 2 N HCl (0.5 mL) over 20 min. After 1 h, the clear solution was treated again with 37% HCHO (2.20 mL) followed by 1 M $NaCNBH_3$ in THF (0.9 mL) and 2 N HCl (0.5 mL) added slowly over 20 min. After 18 h the reaction was quenched with H_2O (3 mL) and the mixture stirred for 15 min. Solvents were removed by rotary evaporation, and the residue was purified by chromatography on silica gel wih solvent D as the eluent. The first fraction eluted from the column (TLC: R_f 0.28; silica gel, solvent A) consisted of unchanged **6** (23 mg, 13% recovery); the second fraction (TLC: R_f 0.22; silica gel, solvent A), after being recrystallized from boiling EtOH, afforded **8** as a beige powder (57 mg, 31% yield); mp 218–221 °C; IR (KBr) ν 3400, 3100, 2950, 1630, 1605, 1570, 1550, 1500 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.53 (s, 3H, N-Me), 3.68 (s, 3H, 5′-OMe), 3.70 (s, 3H, 2′-OMe), 4.22 (s, 2H, CH_2N), 5.89 (br s, 2H, NH_2), 6.62 (m, 1H, 3′- or 4′-H), 6.88 (m, 2H, 3′- or 4′-H and 6-H, overlapping), 6.96 (m, 1H, 6′-H), 7.12 (m, 1H, 7-H), 7.33 (m, 1H, 8-H). Anal. ($C_{18}H_{21}N_5O_2$·0.3H_2O) C, H, N.

2,4-Diamino-5-[(*N*-methyl-3′,4′,5′-trimethoxyanilino)-methyl]quinazoline (9). A stirred mixture of **7** (108 mg, 0.30 mmole) and 37% HCHO (0.30 mL, 0.30 mmol) in MeCN (10 mL) was treated at room temperature with 1 M $NaCNBH_3$ in THF (0.97 mL) followed by slow addition of 2 N HCl (0.1 mL) over 10 min. The resulting clear solution was stirred for 1 h, treated with H_2O (3 mL), stirred for another 15 min, and concentrated to dryness by rotary evaporation. The residue was purified by chromatography on silica gel with solvent D as the eluent. The desired product (TLC: R_f 0.33; silica gel, solvent A) was recrystallized from boiling EtOH to obtain off-white needles (80 mg, 69% yield): mp 201 °C dec; IR (KBr) ν 3450, 3410, 3170, 1625, 1605, 1580, 1560, 1510 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.69 (s, 3H, NMe), 3.57 (s, 3H, 4′-OMe), 3.74 (s, 6H, 3′- and 5′-OMe), 4.47 (s, 2H, CH_2N), 5.95 (s, 2H, 2′- and 6′-H), 6.33 (s, 2H, NH_2), 6.91 (m, 1H, 6-H), 7.16 (m, 1H, 7-H), 7.38 (m, 1H, 8-H), 7.43 (br s, 2H, NH_2). Anal. ($C_{19}H_{23}N_5O_3$·0.1H_2O) C, H, N.

2,4-Diamino-5-(2-phenylethynyl)quinazoline (16). A mixture of **14** (1.7 g, 5.9 mmol), phenylacetylene (0.65 mL, 6.0 mmol), $PdCl_2$ (104 mg, 0.59 mmol), triphenylphosphine (309 mg, 1.18 mmol), CuI (50 mg, 0.59 mmol), Et_3N (2.5 mL), and MeCN (70 mL) was refluxed under argon for 5 h. The reaction mixture was cooled, and the precipitated solid was collected. The filtrate was concentrated to ca. 15 mL by rotary evaporation and chilled, and the precipitate was filtered. The combined solids were purified by chromatography on silica gel with solvent D as the eluent. The desired product (TLC: R_f 0.45, silica gel, solvent A) was a yellow semisolid, which upon recrystallization from boiling EtOH–H_2O yielded yellow needles (323 mg, 21%): mp 188–189 °C; IR (KBr) ν 3420, 3370, 3310, 3160, 1655, 1595, 1560, 1470 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 6.18 (br s, 2H, NH_2), 7.24 (d, 1H, 6- or 7-H), 7.27 (d, 1H, 6- or 7-H), 7.42 (br s, 2H, NH_2), 7.45–7.48 (m, 4H, 8-H, 3′-H, 4′-H, and 5′-H, overlapping), 7.59–7.60 (m, 2H, 2′- and 6′-H). Anal. ($C_{16}H_{12}N_4$·0.5H_2O) C, H, N.

2,4-Diamino-5-(*cis*-2-phenylethenyl)quinazoline (17). A mixture of **16** (90 mg, 0.35 mmol) and 10% Pd–C (25 mg) in 70% AcOH (12 mL) was hydrogenated in a Parr apparatus (55 lb/in.^{2} initial pressure) for 7 h and then filtered through a Celite pad. After washing with 70% AcOH, the combined filtrate and wash solution were concentrated to dryness by rotary evaporation, and the residue was purified by chromatography on silica gel with solvent B as the eluent. The desired product (TLC: R_f 0.33, silica gel, solvent A) was collected as a yellow solid. Recrystallization from 95% EtOH afforded **17** as a white powder (54 mg, 58% yield): mp 246–248 °C; IR (KBr) ν 3370, 3160, 1685, 1655, 1605, 1510 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 6.93 (d, 1H, J = 12 Hz, 9- or 10-H), 6.99 (d, 1H, 6- or 8-H), 7.00–7.02 (m, 2H, 2′- and 6′-H), 7.10 (d, J = 12 Hz, 1H, 9- or 10-H), 7.14–7.16 (m, 3H, 3′-, 4′-, and 5′-H), 7.36 (d, 1H, 6- or 8-H), 7.64 (t, 1H, 7-H). Anal. ($C_{16}H_{16}N_4$·0.1H_2O) C, H, N.

2,4-Diamino-5-(2-phenylethyl)quinazoline (18). To increase its solubility and minimize product loss due to adsorption to the catalyst, **16** (200 mg, 0.77 mmol) was first converted to its trifluoroacetate salt by dissolving it in trifluoroacetic acid (5 mL) and evaporating the solution to thorough dryness *in vacuo* (0.05 Torr, <30 °C bath temperature). The residue was dissolved directly in DMF (15 mL), 10% Pd–C (200 mg) was added, and the suspension was hydrogenated in a Parr apparatus (45 lb/in.^{2} initial pressure) for 20 h. The mixture was filtered through Celite, using DMF to wash the pad, and the combined filtrate and wash solution were concentrated to dryness by rotary evaporation. The residue was chromatographed on silica gel with solvent D as the eluent, and the desired product (TLC: R_f 0.28, silica gel, solvent A) was recrystallized from hot i-PrOH to obtain the trifluoroacetate salt of **18** as white needles (77 mg, 37% yield): mp 199–200 °C; IR (KBr) ν 3420 br, 3190, 1685, 1665, 1610, 1570 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.86 (t, 2H, CH_2), 3.43 (t, 2H, CH_2), 7.16–7.18 (m, 4H, 6-H, 3′-H, 4′-H, and 5′-H, overlapping), 7.23–7.27 (m, 2H, 7- and 8-H), 7.59 (t, 2H, 2′- and 6′-H), 8.30 (br s, 2H, NH_2). Anal. ($C_{16}H_{16}N_4$·CF_3CO_2H·0.3H_2O) C, H, N.

2,4-Diamino-5-[2-(2′,5′-dimethoxyphenyl)ethynyl]-quinazoline (19). A mixture of **14** (600 mg, 2.09 mmol), 2,5-dimethoxyphenylacetylene (35 mg, 2.16 mmol),[21] $PdCl_2$ (30 mg, 0.17 mmol), triphenylphosphine (91 mg, 035 mmol), CuI (15 mg, 0.08 mmol), and Et_3N (0.7 mL) in MeCN (30 mL) was refluxed under argon for 4 h. The clear red solution was cooled, and the precipitated solid was collected, washed with cold MeCN, and dried under reduced pressure. Chromatography on silica gel with solvent D as the eluent afforded the desired product (TLC: R_f 0.38, silica gel, solvent A), which after recrystallization from EtOH was obtained as yellow needles (286 mg, 42%): mp 215–216 °C; IR (KBr) ν 3460, 3330, 1650, 1620, 1565, 1545 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 3H, 5′-OMe), 3.85 (s, 3H, 2′-OMe), 6.08 (br s, 2H, NH_2), 6.98–7.04 (m, 2H, 3′- and 4′-H), 7.09 (d, 1H, 6′-H), 7.21 (d, 1H, 6-H), 7.24 (d, 1H, 7-H), 7.43 (t, 1H, 8-H), 7.84–8.00 (br m, 2H, NH_2). Anal. ($C_{18}H_{16}N_4O_2$·0.2H_2O) C, H, N.

2,4-Diamino-5-[2-(2′,5′-dimethoxyphenyl)ethyl]quinazoline (10). To increase its solubility and improve recovery from the the catalyst after reduction, **19** (100 mg, 0.31 mmol) was converted to a salt by dissolving it in trifluoroacetic acid (3 mL) and evaporating the solution to thorough dryness in vacuo (0.05 Torr, <30 °C bath temperature). The residue was dissolved in DMF (6 mL), 10% Pd–C (200 mg) was added, and the suspension was hydrogenated in a Parr apparatus at 25 lb/in.^{2} initial pressure) for 1 h. The mixture was filtered through Celite, the pad was washed with DMF, and the filtrate and wash solution were combined and concentrated to dryness by rotary evaporation. The residue was purified by chromatography on silica gel using solvent B as the eluent. The desired product (TLC: R_f 0.30; silica gel, solvent A) was collected and recrystallized from hot MeOH to obtain a partial trifluoroacetate salt of **10** as white needles (92 mg, 71% yield): mp 220–223 °C; IR (KBr) ν 3440 br, 1690, 1640, 1605, 1500 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.80 (t, 2H, CH_2), 3.17 (t, 2H, CH_2), 3.67 (s, 3H, 5′-OMe), 3.76 (s, 3H, 2′-OMe), 6.75 (m, 1H, 4′-H), 6.81 (d, 1H, 3′-H), 6.87–6.89 (m, 3H, NH_2 and 6′-H, overlapping), 7.09 (d, 1H, 6-H), 7.21 (d, 1H, 7-H), 7.52 (m, 1H,

8-H, 7.80 (br s, 2H, NH_2). Anal. ($C_{18}H_{20}N_4O_2 \cdot 0.55CF_3CO_2H \cdot H_2O$) C, H, N.

2,4-Diamino-5-[2-(3′,4′,5′-trimethoxyphenyl)ethynyl]-quinazoline (20). A mixture of **15** (600 mg, 2.1 mmol), (3,4,5-trimethoxyphenyl)acetylene (481 mg, 2.51 mmol),[21] $PdCl_2$ (41 mg, 0.21 mmol), triphenylphosphine (109 mg, 0.41 mmol), CuI (20 mg, 0.1 mmol), and Et_3N (0.7 mL) in MeCN (30 mL) was refluxed under argon for 18 h, then cooled, and filtered. The solid was purified by chromatography on silica gel with solvent D as the eluent. The desired product (TLC: R_f 0.45; silica gel, solvent A) was recrystallized from MeOH–H_2O to obtain yellow needles (221 mg, 30% yield): mp 219–220 °C; IR (KBr) ν 3475, 3350, 3140, 1615, 1590, 1560, 1500 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.63 (s, 3H, 4′-OMe), 3.81 (s, 6H, 3′- and 5′-OMe), 6.13 (br s, 2H, NH_2), 6.91 (s, 2H, 2′- and 6′-H), 7.22–7.27 (m, 2H, 6- and 7-H), 7.42 (br s, 2H, NH_2), 7.46 (t, 1H, 8-H). Anal. ($C_{19}H_{18}N_4O_3 \cdot 0.3H_2O$) C, H, N.

2,4-Diamino-5-[2-(3′4′,5′-trimethoxyphenyl)ethyl]-quinazoline (11). To increase its solubility, **20** (120 mg, 0.34 mmol) was dissolved in trifluoroacetic acid (5 mL) and the solution was evaporated to thorough dryness in vacuo (0.05 Torr, <30 °C bath temperature). The residue was dissolved in DMF (10 mL), 10% Pd–C (200 mg) was aded, and the suspension was hydrogenated in a Parr apparatus (25 lb/in.^{2} initial pressure) for 1 h. The mixture was filtered through Celite, the pad was washed with DMF, the combined filtrate and washings were concentrated to dryness by rotary evaporation, and the residue was chromatographed on silica gel with solvent D as the eluent. The desired product (TLC: R_f 0.30; silica gel, solvent A) was obtained as a yellow powder, which on recrystallization from boiling ethanol afforded the trifluoroacetate salt of **11** as beige needles (66 mg, 34%): mp 200–203 °C; IR (KBr) ν 3430, 3390, 3170, 2930, 1665, 1590, 1510 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.79 (t, 2H, CH_2), 3.42 (t, 2H, CH_2), 3.58 (s, 3H, 4′-OMe), 3.69 (s, 6H, 3′- and 5′-OMe), 6.45 (s, 2H, 2′- and 6′-H), 7.20 (d, 1H, 6-H), 7.27 (d, 1H, 7-CH), 7.62 (m, 3H, 8-H and NH_2, overlapping), 8.25 (br s, 2H, NH_2). Anal. ($C_{19}H_{27}N_4O_3 \cdot CF_3CO_2H \cdot 0.3H_2O$) C, H, N.

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References

(1) Ashton, W. T.; Hynes, J. B. Synthesis of 5-substituted quinazolines as potential antimalarial agents. *J. Med. Chem.* **1973**, *16*, 1233–1237.
(2) Hynes, J. B.; Ashton, W. T.; Bryansmith, D.; Freisheim, J. H. Quinazolines as inhibitors of dihydrofolate reductase. 2. *J. Med. Chem.* **1974**, *17*, 1023–1025.
(3) Harris, N. V.; Smith, C.; Bowden, K. Antifolate and antibacterial activities of 5-substituted 2,4-diaminoquinazolines. *J. Med. Chem.* **1990**, *33*, 434–444.
(4) Ercikan E.; Waltham, M.; Dicker, A.; Schweitzer, B.; Bertino, J. R. Effect of codon 22 mutations on substrate and inhibitor binding for human dihydrofolate reductase. *Adv. Exp. Med. Biol.* **1993**, *338*, 515–519.
(5) Melera, P. W; Davide, J. P.; Hession, C. A.; Scotto, K. W. Phenotypic expression in *Escherichia coli* and nucleotide sequence of two Chinese hamster lung cell cDNAs encoding different dihydrofolate reductases. *Mol. Cell. Biol.* **1984**, *4*, 38–48.
(6) Dicker, A. P.; Volkenandt, M.; Schweitzer, B. I.; Banerjee, D.; Bertino, J. R. Identification and characterization of a mutation in the dihydrofolate reductase gene from the methotrexate-resistant Chinese hamster ovary cell line Pro^{-3} MTX^{RIII}. *J. Biol. Chem.* **1990**, *265*, 8317–8321.
(7) Simonsen, C. C.; Levinson, A. D. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2495–2499.
(8) Mills, J. *Pneumocystis carinii* and *Toxoplasma gondii* infections in patients with AIDS. *Rev. Infect. Dis.* **1986**, *8*, 1001–1011.
(9) Kovacs, J. A.; Allegra, C. A.; Swan, J. C.; Drake, J. C.; Parrillo, J. E.; Chabner, B. A; Masur, H. Potent antipneumocystis and antitoxoplasma activities of piritrexim, a lipid-soluble antifolate. *Antimicrob. Agents Chemother.* **1988**, *32*, 430–433.
(10) Allegra, C. J.; Chabner, B. A.; Tuazon, C. U.; Ogata-Arakaki, D.; Baird, B.; Drake, J. C.; Simmons, J. T.; Lack, E. E.; Shelhamer, J. H.; Balis, F.; Walker, R.; Kovacs, J. A.; Lane, H. C.; Masur, H. Trimetrexate for the treatment of *Pneumocystis carinii* pneumonia in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **1987**, *317*, 978–985.
(11) Masur, H.; Polis, M. A.; Tuazon, C. U.; Ogata-Arakaki, D.; Kovacs, J. A.; Katz, D.; Hilt, D.; Simmons, T.; Feuerstein, I.; Lundgren, B.; Lane, H. C.; B. A. Chabner, B. A.; Allegra, C. J. Salvage trial of trimetrexate-leucovorin for the treatment of cerebral toxoplasmosis. *J. Infect. Dis.* **1993**, *167*, 1422–1426.
(12) Sattler, F. R.; Frame, P.; Davis, R.; Nichols, L.; Shelton, B.; Akil, B.; Baughman, R.; Hughlett, C.; Weiss, W.; Boylen, C. T.; van der Horst, C.; Black, J.; Powderly, W.; Steigbigel, R. T.; Leedom, J. M.; Masur, H.; Feinberg, J.; Benoit, S.; Eyster, E.; Gocke, D.; Beck, K.; Lederman, M.; J. Phair, J.; Reichman, R.; Sacks, H. S.; and R. Soiero, R. Trimetrexate and leucovorin versus trimethoprim-sulfamethoxazole for moderate to severe episodes of *Pneumocystis carinii* pneumonia in patients with AIDS: a prospective, controlled multicenter investigation of the AIDS Clinical Trials Group Protocol 029/031. *J. Infect. Dis.* **1994**, *170*, 165–172.
(13) Kovacs, J. A.; Allegra, C. J.; Beaver, J.; Boarman, D.; Lewis, M.; Parrillo, J. E.; Chabner, B.; and H. Masur, H. Characterization of de novo folate synthesis in *Pneumocystis carinii* and *Toxoplasma gondii*: potential for screening therapeutic agents. *J. Infect. Dis.* **1989**, *160*, 312–320.
(14) Southerland, W. M. A molecular model of the folate binding site of *Pneumocystis carinii* dihydrofolate reductase. *J. Comput.-Aided Mol. Design* **1994**, *8*, 113–122.
(15) Hynes, J. B.; Pathak, A.; Panos, C. H.; and Okeke, C. C. Direct synthesis of 2,4-diaminoquinazolines from 2-fluorobenzonitriles. *J. Heterocycl. Chem.* **1988**, *25*, 1173–1177.
(16) Hynes, J. B.; Tomazic, A.; Parrish, C. A.; Fetzer, O. S. Further studies on the synthesis of quinazolines from 2-fluorobenzonitriles. *J. Heterocycl. Chem.* **1991**, *28*, 1357–1363.
(17) Elslager, E. F.; Johnson, J. L.; and Werbel, L. M. Folate antagonists. 20. Synthesis and antitumor and antimalarial properties of trimetrexate and related 6-[(phenylamino)methyl]-2,4-quinazolinediamines. *J. Med. Chem.* **1983**, *26*, 1753–1760.
(18) Taylor, E. C. New pathways from pteridines. Design and synthesis of a new class of potent and selective antitumor agents. *J. Heterocycl. Chem.* **1990**, *27*, 1–12 (1990).
(19) Gangjee, A.; Devraj, R.; Lin, F.-T. Synthesis of 2,4-diamino-5,-10-dideaza non-classical antifolates. *J. Heterocycl. Chem.* **1991**, *28*, 1747–1751.
(20) Kato, M.; Kido, F.; Wu, M. D.; Yoshikoshi, A. Synthetic study of colchicine. *Bull. Chem. Soc. Jpn.* **1974**, *47*, 1516–1521.
(21) Buckle D. R.; Rockell, C. J. M. A versatile two-stage synthesis of 2-substituted benzo[*b*]furans from (2-methoxyphenyl)ethynes. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2443–2446.
(22) Schweitzer, B. I.; Srimatkandada, S.; Gritsman, H.; Sheridan, R.; Venkataraghavan, R.; Bertino, J. R. Probing the role of two hydrophobic active site residues in the human dihydrofolate reductase by site-directed mutagenesis. *J. Biol. Chem.* **1989**, *264*, 20786–20795.
(23) Piper, J. R.; Johnson, C. A.; Hosmer, C. A.; Carter, R. L.; Pfefferkorn, E. R.; Borotz, S. E.; Queener, S. F. Lipophilic antifolates as candidates against opportunistic infections. *Adv. Exp. Biol. Med.* **1993**, *338*, 429–433.
(24) Queener, S. F.; Bartlett, M. S.; Jay, M. A.; Durkin, M. M.; Smith, J. W. Activity of lipid-soluble inhibitors of dihydrofolate reductase against *Pneumocystis carinii* in culture and in a rat model of infection. *Antimicrob. Agents Chemother.* **1987**, *31*, 1323–1327.
(25) Chio, L.-C.; Queener, S. F. Identification of highly potent and selective inhibitors of *Toxoplasma gondii* dihydrofolate reductase. *Antimicrob Agents Chemother.* **1993**, *37*, 1914–1923.

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