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Preliminary in vitro studies on two potent, water-soluble trimethoprim analogues with exceptional species selectivity against dihydrofolate reductase from *Pneumocystis carinii* and *Mycobacterium avium*

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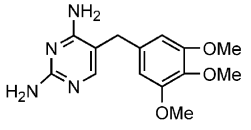
Abstract—2,4-Diamino-5-[3′,4′-dimethoxy-5′-(5-carboxy-1-pentynyl)]benzylpyrimidine (**6**) and 2,4-diamino-5-[3′,4′-dimethoxy-5′-(4-carboxyphenylethynyl)benzylpyrimidine (**7**) were synthesized from 2,4-diamino-5-(5′-iodo-3′,4′-dimethoxybenzyl)pyrimidine (**9**) via a Sonogashira reaction with appropriate acetylenic esters followed by saponification, and were tested as inhibitors of dihydrofolate reductase (DHFR) from *Pneumocystis carinii* (Pc), *Toxoplasma gondii* (Tg), *Mycobacterium avium* (Ma), and rat in comparison with the widely used antibacterial agent 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (trimethoprim, TMP). The selectivity index (SI) for each compound was calculated by dividing its 50% inhibitory concentration (IC_{50}) against rat DHFR by its IC_{50} against Pc, Tg, or Ma DHFR. The IC_{50} of **6** against Pc DHFR was 1.0 nM, with an SI of 5000. Compound **7** had an IC_{50} of 8.2 nM against Ma DHFR, with an SI of 11000. By comparison, the IC_{50} of TMP was 12000 nM against Pc, 300 nM against Ma, and 180000 against rat DHFR. The potency and selectivity values of **6** and **7** were not as high against Tg as they were against Pc or Ma DHFR, but nonetheless exceeded those of TMP. Because of the outstanding selectivity of **6** against Pc and of **7** against Ma DHFR, these novel analogues may be viewed as promising leads for further structure–activity optimization.

The well-known antimicrobial agent trimethoprim (TMP, **1**)[1a] is one the most widely prescribed antimicrobial drugs in the world. As an ingredient in cotrimoxazole,[1b] a two-drug combination also containing sulfamethoxazole, TMP is used extensively for the prophylaxis and/or therapy of potentially life-threatening opportunistic infections in patients with AIDS.[2] Although the role of such prophylaxis is less widely accepted outside the AIDS setting, there is nonetheless accruing evidence that it can be useful in the clinical management of cancer patients with certain hematologic malignancies, whose immune system is temporarily compromised as a result of whole-body radiotherapy or the administration of high doses of steroids (e.g., for brain tumors), or who are treated with immunosuppressive drugs like cyclosporin as part of an organ transplantation regimen.[3] Three of the opportunistic diseases prevalent among AIDS patients, and often the first telltale sign of HIV-1 infection, are pneumocystis pneumonia, toxoplasmic encephalitis, and disseminated infection by *Mycobacterium avium* complex (MAC). Pneumocystis pneumonia is caused by the fungal organism *Pneumocystis carinii*, whereas cerebral toxoplasmosis generally results from latent activation of *Toxoplasma gondii* spores typically transmitted to humans earlier in life by domestic pets or farm animals. Several recent examples of the clinical efficacy of prophylaxis with cotrimoxazole to prevent *P. carinii* and *T. gondii* infections in the non-AIDS setting are of interest.[4]



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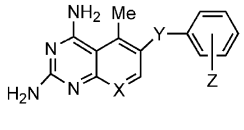

2: X = CH, Y = CH_2NH, Z = 3′,4′,5′-$(OMe)_3$
3: X = N, Y = CH_2, Z = 2′,5′-$(OMe)_2$

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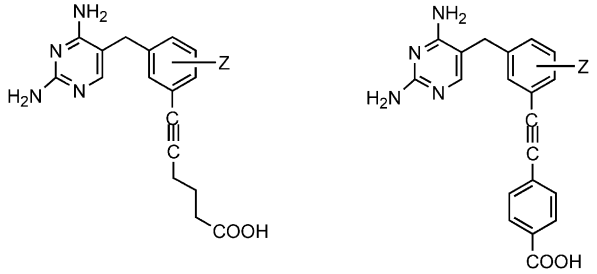
Trimethoprim acts by blocking the action of dihydrofolate reductase (DHFR),[5] which catalyzes hydride transfer from NADPH to dihydrofolate. The resulting tetrahydrofolate product is a co-factor in the biosynthesis of the purine and pyrimidine building blocks required for RNA and DNA synthesis. The 5,10-methylene derivative tetrahydrofolate is the natural substrate for a second key enzyme, thymidylate synthase, whose role is to convert 2′-deoxyuridylate (dUMP) to 2′-deoxythymidylate (dTMP) for subsequent incorporation into DNA. The other product of this reaction is dihydrofolate, and the latter has to be reduced back to tetrahydrofolate by DHFR in order for the process to continue. In the context of antimicrobial chemotherapy, the potency of TMP is generally not sufficient by itself to bring the number of infectious organisms down to a low enough level to assure success. For this reason, a sulfa drug is co-administered to block a second enzyme, dihydropteroate synthetase, which is used for the de novo synthesis of folates by many lower organisms including *P. carinii*, *T. gondii*, and *M. avium*, but is absent in mammals.[6] At the present time the only sulfa drug used clinically in combination with TMP, other than sulfamethoxazole, is dapsone.[7] A major drawback to the use of TMP–sulfa combinations in immunocompromised patients is the occurrence of hypersensitivity reactions to the sulfa drug that can be severe enough to require discontinuation of treatment when the side effects cannot be managed with steroids, especially since these can actually exacerbate the infection.[8] More potent DHFR inhibitors than TMP, such as trimetrexate (TMX, **2**)[9] or piritrexim (PTX, **3**),[10] do not require potentiation by a sulfa drug. However, because these potent antifolates, unlike TMP, are not selective for non-mammalian versus mammalian DHFR they can produce dangerous hematologic side effects. Thus they require administration of a protective or 'rescue' agent in the form of leucovorin (LV, 5-formyltetrahydrofolate) in order to be clinically useful. While the TMX-LV combination has aroused clinical interest for the treatment of acute pneumocystis pneumonia in AIDS patients who cannot tolerate sulfa drugs (or other popular agents such as aerosolized pentamidine), even more vigilant clinical monitoring needs to be practiced with this regimen than with TMP–sulfamethoxazole or TMP–dapsone. Thus DHFR inhibitors that are more potent than TMP and do not require co-administration of a sulfa drug, while being safe to use in humans without LV, are of considerable therapeutic interest.

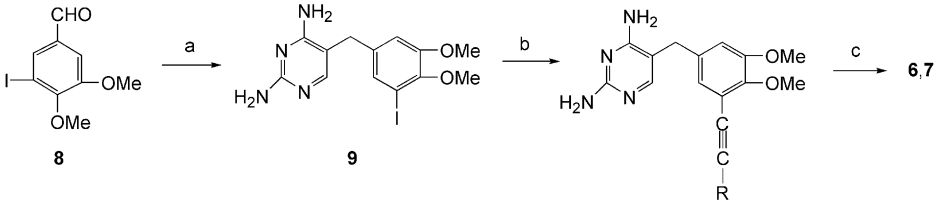
As part of a larger study aimed at the discovery of newer DHFR inhibitors that fulfill the elusive criterion of blending into a single molecule the selectivity of TMP and the potency of TMX or PTX, we recently synthesized and tested a number of 2,4-diamino-5-(2′,5′-disubstituted benzyl)pyrimidines containing a COOH group linked to the benzyl ring via a spacer.[11–13] The 2′,5′-substitution pattern was chosen to differentiate these compounds from TMP (selective but not potent) and bring them closer in structure to piritrexim (potent but not selective). Two of the more interesting compounds in this group were the 5′-(5-carboxy-1-pentynyl) analogue **4**[11] and the 5′-(4-carboxyphenylethynyl) analogue **5**.[12]



4: Z = 2′-OMe
6: Z = 3′,4′-$(OMe)_2$

5: Z = 2′-OMe
7: Z = 3′,4′-$(OMe)_2$

Armed with knowledge of the pioneering work of Kuyper et al. on TMP analogues with a carboxyalkyl group on the 5′-oxygen as inhibitors of *Eschericia coli* DHFR,[14] we could not pass up the opportunity to examine the effect of 3′,4′-dimethoxy-5′-(5-carboxy-1-pentynyl)- and 5′-(4-carboxyphenylethynyl) substitution on Pc, Tg, and Ma DHFR inhibition. Thus, extending the method we had already followed to prepare **4** and **5**, we synthesized the 3′,4′,5′-trisubstituted analogues **6** and **7** from 5-iodo-3,4-dimethoxybenzaldehyde (**8**).[15] As shown in Scheme 1, condensation with 3-morpholinopropionitrile in the presence of NaOEt, followed by aniline hydrochloride, and finally guanidine[16] afforded the diaminopyrimidine **9**. A Pd(II)-catalyzed Sonogashira coupling reaction[17] was then used to obtain alkyne esters **10** and **11** from **9** and benzyl 5-hexynoate and methyl 4-ethynylbenzoate, respectively. Hydrolysis of the esters with NaOH in DMSO followed by preparative HPLC on C_{18} silica gel yielded the desired acids **6** and **7**. The identity and purity of the products was established by microchemical analysis as well as from their IR and ^{1}H NMR spectra.



10: $(CH_2)_3CO_2Bn$
11: $C_6H_4(4\text{-}CO_2Et)$

Scheme 1. (a) (i) 3-morpholinopropionitrile, NaOEt; (ii) $PhNH_2{\cdot}HCl$; (iii) $H_2NC(=NH)NH_2$; (b) $RC{\equiv}CH$, $(Ph_3P)_2PdCl_2$, $(Ph_3P)_3CuBr$, Et_3N; (c) NaOH, DMSO.

Table 1. Inhibition of Pc, Tg, Ma, and rat DHFR by 2,4-diamino-5-(3',4'-dimethoxy-5'-substituted benzyl]pyrimidines with a carboxyalkynyl or carboxyphenylalkynyl group in the side chain

	IC_{50} (nM)[a]				Selectivity Index (SI)[b]		
Cmpd	Pc	Tg	Ma	rat	Pc	Tg	Ma
4	28 (25–30)	32 (27–37)	7.8 (6.4–9.5)	2200 (1900–2500)	79 (63–100)	69 (52–91)	280 (200–380)
5	1300 (0.65–2.5)	340 (0.20–0.55)	3.7 (0.0029–0.0046)	8200 (7100–9300)	6.3 (2.8–14)	24 (13–47)	2200 (1500–3200)
6	1.0 (0.82–1.2)	34 (30–39)	2.4 (0.0021–0.0027)	5000 (4300–5800)	5000 (3600–7100)	150 (110–190)	2100 (2000–2800)
7	1200 (990–1400)	420 (300–590)	8.2 (6.9–9.8)	88000 (57000–140000)	73 (41–140)	210 (97–470)	11000 (5800–20000)
TMP[c]	13000 (10000–16000)	2800 (2400–3300)	300 (260–350)	180000 (160000–210000)	14 (10–20)	65 (48–87)	610 (460–810)
TMX[d]	47 (34–66)	16 (8–30)	1.5 (1.3–1.7)	8.0 (7.0–9.2)	0.017 (0.11–0.27)	0.50 (0.23–1.2)	5.3 (4.1–7.1)
PTX[e]	13 (9.0–17)	4.3 (4.0–4.6)	0.61 (0.53–0.70)	3.3 (2.9–3.9)	0.26 (0.17–0.42)	0.76 (0.63–0.97)	5.4 (4.1–7.2)

[a] Numbers in parentheses are the 95% confidence intervals, rounded off to two significant figures and are based on IC_{50} values similarly rounded off to two figures. The difference in IC50 between rat liver DHFR and each of the parasite enzymes was statistically significant at $P<0.01$ (Welsh's *t*-test). Data for **4** and **5** shown for comparison are from refs. 13 and 14 respectively.

[b] SI = IC_{50}(rat DHFR)/IC_{50}(Pc, Tg, or Ma DHFR). Numbers in parentheses are rounded off to two figures, and represent a range calculated by dividing the lower end of the 95% confidence range for the IC_{50} against rat DHFR by the upper end of the 95% confidence range for the IC_{50} against Pc, Tg, or Ma DHFR.

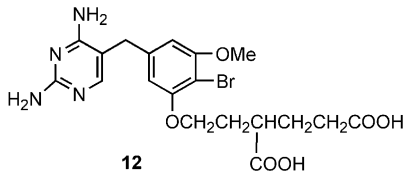
[c] TMP = 2,4-diamino-5-(3′,4′,5′-trimethoxybenzylpyrimidine); data given for comparison are from ref. 14.

[d] TMX = 2,4-diamino-5-methyl-6-(3′,4′,5′-trimethoxyanilino)-methylquinazoline.

[e] PTX = 2,4-diamino-5-methyl-6-(2′,5′-dimethoxybenzyl)pyrido[2,3-*d*]pyrimidine; data given for comparison are from ref. 14.

The ability of **6** and **7** to inhibit Pc, Tg, Ma, and rat DHFR was determined by the standardized method in our previous work.[18] The assay is based on measurement of the change in absorbance at 340 nm when dihydrofolate is reduced to tetrahydrofolate in the presence of NADPH. The results are presented in Table 1.

Whereas the IC_{50} of **4** against Pc and Ma DHFR was previously found to be 28 nM and 7.8 nM, respectively, the corresponding values for the 3′,4′,5′-trisubstituted analogue **6** proved to be ca. 3-fold lower (Table 1), indicating that the latter structure is more favorable for binding to these enzymes. More significantly, binding to rat DHFR was also decreased ca. 2-fold, resulting in a *>10-fold improvement in selectivity against both enzymes*. By comparison, the IC_{50} against Tg DHFR was unaffected, and selectivity was only marginally enhanced. The 5000-fold selectivity of **6** against Pc versus rat DHFR is considerably higher than we have encountered with any of our 2′,5′-disubstituted inhibitors with a terminal COOH group in the side chain, rivaling only that of the *dicarboxylic* acid **12**, which is claimed to have 4300-fold selectivity for Pc versus human DHFR in a patent by Kompis et al.[19] Compound **12** is structurally related to 2,4-diamino-5-(4′-bromo-3′,5′-dimethoxybenzyl)pyrimidine (brodimoprim, BMP), which has an overall spectrum of antibacterial activity not dissimilar from that of TMP but displays more favorable cell penetration and pharmacokinetics.[20]



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With regard to phenylalkynyl substitution, the IC_{50} of **7** against Pc and Tg DHFR was not substantially different from that of **5**. However binding to Ma DHFR was enhanced ca. 2-fold while binding to rat DHFR was decreased 11-fold. As a consequence, the selectivity index of **7** increased 5-fold to 11,000, making this the most selective TMP analogue we have tested to date against the Ma enzyme.[21]

It should be noted that **6** was 12,000 times more potent than TMP against Pc DHFR, and that its potency actually exceeded that of TMX and PTX by a factor of at least 10. However, in contrast to the latter drugs, which are much more potent against rat than Pc DHFR and thus may be said to be 'reverse-selective', **6** shows a level of binding selectivity at least 300-fold greater than that of TMP. A further advantage of both **6** and **7** over TMP, TMX, and PTX is that they are soluble in water at physiologic pH, and thus are more suitable for parenteral administration.[26]

The fact that a DHFR inhibitor is potent against the enzyme does not, of course, mean that it will have an antifolate effect on intact cells. To address this issue, the effect of **6** on proliferation of Pc cells was compared with that of TMP in a luciferase-based bioluminescence assay of ATP.[23–25] Treatment of three different Pc isolates (from immunosuppressed rats) with 100 μg/mL (250 μM) of **6** in RPMI 1640 medium containing 20% calf serum for 72 h resulted in a mean T/C (treated/control) value of 40%, whereas the T/C for 100 μg/mL (180 μM) of TMP under the same conditions was 22%. As would be expected, lower drug concentrations (e.g., 10 μg/mL) or shorter treatment times (e.g., 48 h) were less effective for both drugs. Thus, it was gratifying that, while its molar potency against intact Pc was slightly

lower that of TMP, **6** nonetheless had the ability to enter Pc cells *despite the presence of a free COOH group*.

In summary, our results indicate that, where binding and selectivity for Pc DHFR are concerned, 5′-(5-carboxy-1-pentynyl) substitution is more favorable when the benzyl ring is 3′,4′,5′-trisubstituted than when it is 2′,5′-disubstituted. Where binding to Ma DHFR is concerned, both 5′-(5-carboxy-1-pentynyl) and 5′-(4-carboxyphenylethynyl) substitution are more conducive to binding and selectivity when the benzyl ring is 3′,4′,5′-trisubstituted. Compounds **6** and **7** have the highest species selectivity for Pc and Ma DHFR respectively, of any 2,4-diamino-5-benzylpyrimidine inhibitors of these enzymes we have synthesized and tested to date. For this reason these novel analogues may be viewed as novel leads for further structure–activity optimization of DHFR binding, and ultimately cell penetration, tissue distribution, metabolism, and pharmacokinetics.

Acknowledgements

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21. After completing the synthesis and testing of **7** we discovered that this structure was claimed as part of a patent on TMP analogues as antimicrobials against gram-negative bacteria, and *P. carinii*.[22] However, only the synthesis of this compound was described in the patent, and no data on binding to either Pc or Ma were disclosed.
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24. We are grateful to Dr. Melanie Cushion, Dept. of the VA Medical Center, University of Cincinnati, for kindly performing these assays.
25. Although assays using **6** against intact Ma cells have not yet been performed, it may be noted that Dr. Clark Inderlied (Children's Hospital Medical Center, Los Angeles, CA) recently found that the 2′,5′-disubstituted analogue **4** inhibits the growth of human isolates of this organism in culture at concentrations of 4-32 mg/mL depending on the isolate, whereas TMP was inactive even at 64 μg/mL.[13]

26. The relative ability of water-soluble TMP analogues containing a COOH group in the side chain to enter cells can be expected to vary substantially depending on the target organism. For example, Kuyper et al.[14] reported that 2,4-diamino-5-[3′,4′-dimethoxy-5′-(5-carboxypentyloxy)]pyrimidine, a structural analogue of **6**, had nearly the same activity as TMP against *S. aureus*, but was 300-fold less active than TMP against *E. coli* organisms in culture even though it had 50-fold greater affinity for *E. coli* DHFR in a cell-free assay.