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Pyrrolo[2,3-*d*]pyrimidines and Pyrido[2,3-*d*]pyrimidines as Conformationally Restricted Analogues of the Antibacterial Agent Trimethoprim

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Abstract—Conformationally restricted analogues of the antibacterial agent trimethoprim (TMP) were designed to mimic the conformation of drug observed in its complex with bacterial dihydrofolate reductase (DHFR). This conformation of TMP was achieved by linking the 4-amino function to the methylene group by one- and two-carbon bridges. A pyrrolo[2,3-*d*]pyrimidine, a dihydro analogue, and a tetrahydropyrido[2,3-*d*]pyrimidine were synthesized and tested as inhibitors of DHFR. One analogue showed activity equivalent to that of TMP against DHFR from three species of bacteria. An X-ray crystal structure of this inhibitor bound to *Escherichia coli* DHFR was determined to evaluate the structural consequences of the conformational restriction.

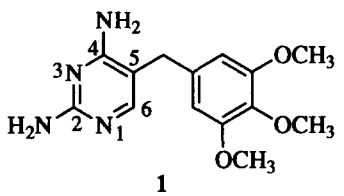
Introduction

The broad-spectrum antibacterial action of trimethoprim (**1**, TMP) results from selective inhibition of bacterial dihydrofolate reductase (DHFR).[1,2] The ratio of TMP K_i values for *Escherichia coli* and human DHFR is about 10,000.[3,4] The drug binds to bacterial DHFR in a conformation significantly different from the conformation bound to human and chicken DHFR as shown by X-ray crystallographic[5–8] and NMR studies.[9–14] That structural information provides a unique opportunity to design conformationally restricted analogues that might have improved selectivity and affinity. Restricting the conformation of TMP to that observed in the bacterial enzyme complex might enhance selectivity, assuming that the bacterial enzyme-bound conformation is unfavorable for binding to vertebrate DHFR. Conformational restriction might also provide an increase in affinity through the entropic effect of pre-organization. Chan and Roth recently reported the design and synthesis of a conformationally restricted TMP analogue in which the *ortho*-position of the phenyl ring is joined to the methylene group with a two-carbon linkage.[15] This paper describes the design, synthesis, and DHFR inhibition of three TMP analogues in which the conformation was constrained by one- and two-carbon bridges from the 4-amino substituent to the methylene group.

Compound Design

Trimethoprim binds to *Lactobacillus casei* and *E. coli* DHFR in a conformation significantly different from that observed in the human and chicken enzymes.[10,16,17] The two conformations are shown in Figure 1. We sought ways to rigidify the conformation of TMP to favor the form of the drug observed in the bacterial DHFR complex without detrimentally affecting enzyme binding.

Linking the 4-amino group of TMP to the methylene moiety appeared to be a viable means of restricting



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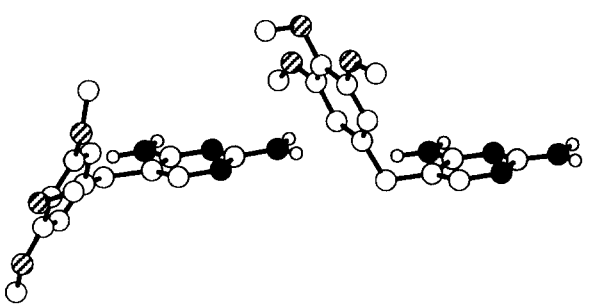


Figure 1. The conformations of TMP in its complex with *E. coli* DHFR (left) and chicken DHFR (right) as determined by X-ray crystallography.[8] Nitrogen atoms are represented as black spheres, carbon atoms are the larger white spheres, oxygens are striped, and hydrogens are small white spheres. Hydrogens on carbon are not shown.

conformation in the desired fashion. TMP is protonated at the pyrimidine ring N-1 position in its complex with DHFR[9,13] and is bound to the *E. coli* enzyme through five hydrogen bonds, as shown in Figure 2. One of the 2-amino hydrogens and the hydrogen of N-1 serve as donors in hydrogen bonds to the carboxylate of Asp-27. The second hydrogen of the 2-amino group interacts with a buried water molecule, and both hydrogens of the 4-amino group are involved in hydrogen bonds to backbone carbonyl groups of the protein. Thus, connecting the 4-amino group to the methylene would disrupt the interaction with the carbonyl group of Ile-94 of the *E. coli* enzyme. However, the contribution of that hydrogen bond to the enzyme affinity of the drug was unknown, and evidence concerning the interaction between the substrate dihydrofolic acid and the enzyme active site suggested that the enzyme might accommodate the 4-amino-to-methylene linkage under consideration. A variety of data[18–20] including recent X-ray crystallographic work,[21] strongly suggest that the pteridine ring of dihydrofolate binds to DHFR as shown in Figure 3. The pteridine ring system of the substrate is linked to the enzyme through hydrogen bonds that are analogous to four of those observed for TMP. However, a hydrogen-bonding interaction with the backbone carbonyl oxygen of Ile-94 is not possible in the substrate complex, and the 6- and 7-positions of the pterin ring system occupy a region of the active site that would be occupied by the linker atoms of the proposed analogues of TMP. Thus, the similarity of the substrate–enzyme interactions to those envisioned for the 4-amino-to-methylene bridged analogues provided some support for this approach to conformational restriction.

Compounds **2–4** were designed based on the above considerations. Molecular modeling of structures **2–4** suggested that each type of 4-amino-to-methylene bridge provided conformational constraints that were reasonably compatible with the bacterial enzyme-bound conformation of TMP. Minimum energy conformations of **2–4** are shown in Figure 4. In the design of structure **2**, the methyl group on the pyrimidine ring was included to force the adjacent phenyl ring out of the plane of the pyrrolopyrimidine ring system and into a conformation that better matched that of enzyme-bound TMP. The position of the phenyl group with respect to the pyrimidine ring is different among structures **2–4**, but the orientation in each structure was reasonably close to that observed for TMP bound to *E. coli* DHFR. For compounds **3** and **4**, each of the two enantiomers displayed a conformation that was sufficiently similar to that of enzyme-bound TMP to be of interest.

Chemistry

As shown in Scheme 1, thermally induced Fischer indole synthesis[21] was employed for the preparation of pyrrolopyrimidine **2**. 2-Amino-4-hydrazino-6-methylpyrimidine (**5**)[23] was condensed with aldehyde **6**,[24] and

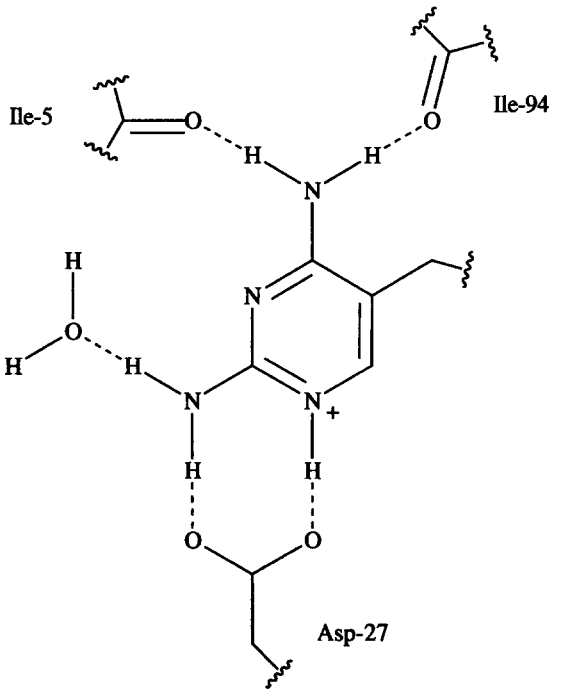


Figure 2. Hydrogen bonds involved in binding of TMP to *E. coli* DHFR as determined by X-ray crystallography.[8]

the resulting hydrazone **7** was cyclized to the desired product in refluxing tetralin.

In the preparation of compound **3** (see Scheme 2), key intermediate **11** was prepared by the methods of Langlois et al.[25] Michael addition of nitromethane to the benzylidenemalonate **8** gave the adduct **9** in good yield. Catalytic reduction of **9** furnished pyrrolidone **10** in 80% yield. The lactim ether **11** was prepared from pyrrolidone **10** using triethyloxonium tetrafluoroborate. Following the work of Granik and Glushkov,[26] pyrroline **11** was condensed with guanidine to provide the dihydropyrrolopyrimidone **12** in 59% yield. To remove the oxo group of **12**, a mesylation/reduction procedure

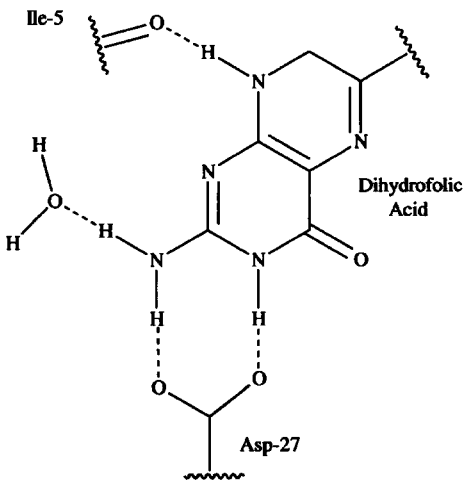
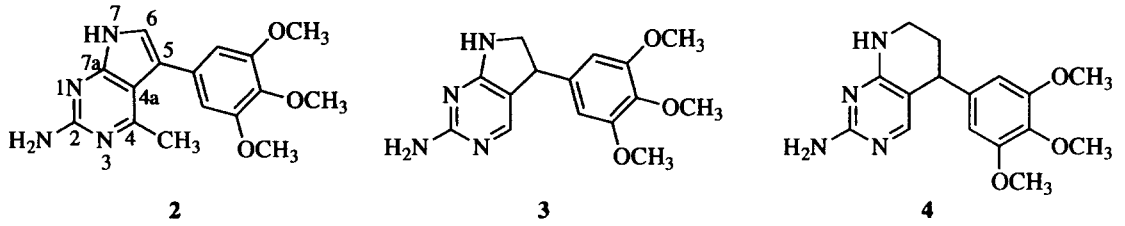


Figure 3. The postulated binding of dihydrofolic acid to the active site of *E. coli* DHFR.[21]



2 3 4

of Nussim and Weinik[27] was employed. Mesylation of **12** was carried out in pyridine, and the product was reduced over Pd on C in hot acetic acid to afford the target compound **3**.

The synthesis of compound **4** is illustrated in Scheme 3. Knoevenagel condensation of 3,4,5-trimethoxyacetophenone and malononitrile gave the ethylidenemalononitrile **14** in good yield. Pyridine **16** was prepared using methodology reported by Baldwin et al.,[28] which involved treatment of **14** with DMF dimethylacetal to give **15** and cyclization of **15** to **16** using HBr in acetic acid. Condensation of pyridine **16** with guanidine provided the diaminopyridopyrimidine **17**. The 4-amino group of **17** was selectively hydrolyzed, and the pyridine ring of the resulting 4-oxo derivative **18** was cleanly reduced under catalytic conditions in trifluoroacetic acid. As in the synthesis of **3** described above, the oxo function of **19** was removed via a mesylation/reduction procedure to provide the target compound **4**.

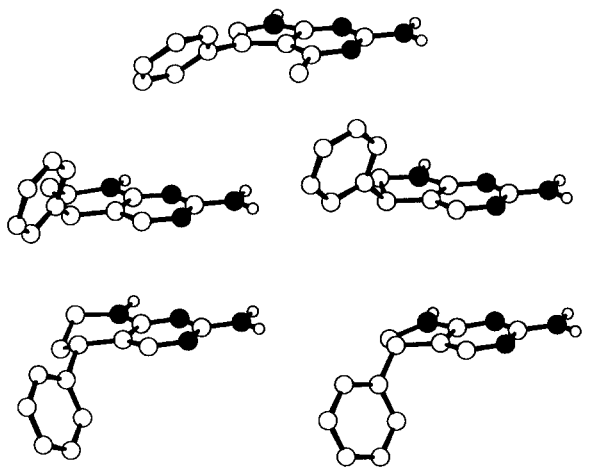


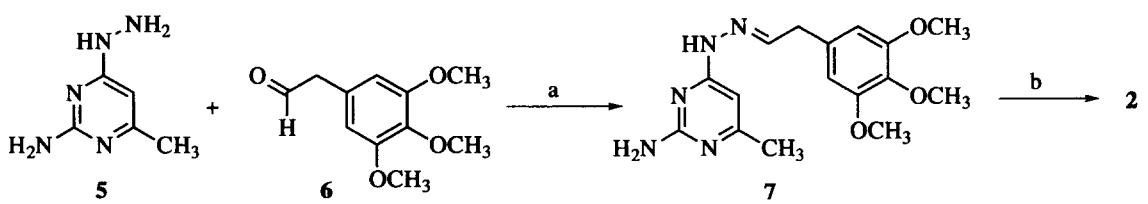
Figure 4. Minimum energy conformations of compounds **2** (top), **3** (middle, enantiomeric pair), and **4** (bottom, enantiomeric pair), calculated using MM2 as implemented in MacroModel Version 3.5X.[22] Nitrogen atoms are shown as black spheres, carbons as white spheres, and hydrogens as smaller white spheres. The methoxy groups and hydrogens attached to carbon are omitted for clarity.

X-ray Crystallography

The crystal structure of compound **2** complexed with *E. coli* DHFR was determined from co-crystallization experiments as described for the corresponding complexes of TMP and related inhibitors.[8] The resolution of the data from the complex with compound **2** extended to 2.8 Å, and the structure was refined to a crystallographic R-value of 16% using PROFFT.[29] The asymmetric unit contained two binary complexes, and the protein structure in each complex was essentially similar to that seen for the crystal structure of the TMP binary complex.[8] The pyrimidine ring of compound **2** was located in the active site in the same way as for the TMP complex, with the 2-amino group and N-1 interacting via hydrogen bonds with Asp-27. The trimethoxyphenyl group of compound **2** was located in a similar environment to that seen for the trimethoxybenzyl group of the TMP complex, but in the present case was positioned slightly nearer to residues 49 and 50.

Results and Discussion

DHFR inhibition data for compounds **1–4** are shown in Table 1. TMP (**1**) exhibited potent inhibition of each of the four species of bacterial DHFR shown in Table 1 and was substantially less active against the vertebrate enzyme, rat liver DHFR. Of the three conformationally restricted analogues that we prepared, pyrrolopyrimidine **2** displayed the most interesting activity. Although 60-fold less active than TMP against *E. coli* DHFR, compound **2** showed inhibitory activities against DHFR from *Staphylococcus aureus*, *Plasmodium berghei*, and *Neisseria gonorrhoeae* that were essentially equivalent to those of TMP. However, since the affinity of compound **2** for rat liver DHFR was



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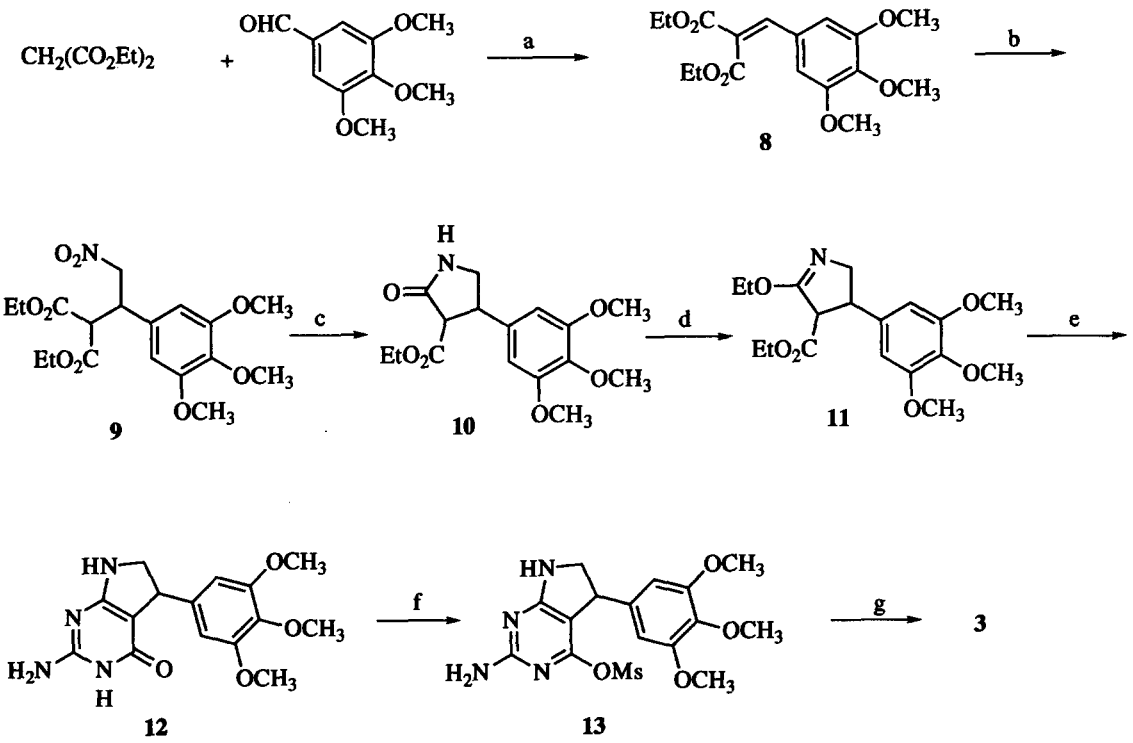
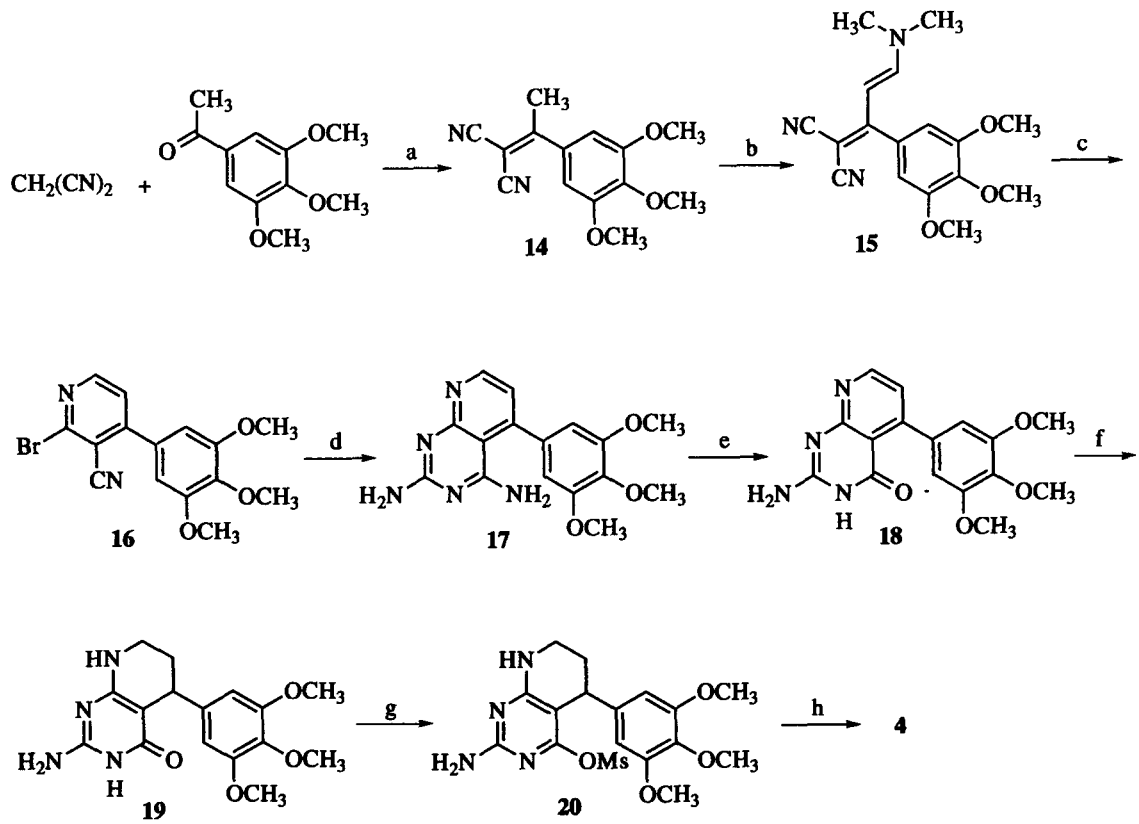
Scheme 1. Reagents and conditions: (a) boiling water; (b) boiling tetralin.



Scheme 2. Reagent and conditions: (a) piperidine, 3,4,5-trimethoxybenzoic acid, toluene; (b) nitromethane, NaOEt; (c) hydrogen, 5% Pd on C; (d) triethyloxonium tetrafluoroborate; (e) guanidine; (f) mesyl chloride, pyridine; (g) hydrogen, 5% Pd on C.



Scheme 3. Reagents and conditions: (a) acetic acid, ammonium acetate; (b) DMF dimethylacetal; (c) HBr, acetic acid; (d) guanidine; (e) NaOH; (f) H_2, PtO_2; (g) MsCl, 2,6-lutidine; (h) H_2, 5% Pd/C.

10-fold higher than that observed for TMP, its selectivity for *S. aureus*, *P. berghei*, and *N. gonorrhoeae* DHFR was less favorable. Compounds **3** and **4** displayed activity against each of the bacterial species of DHFR that was significantly weaker than that of inhibitors **1** and **2**. The weak inhibition of vertebrate DHFR by compounds **3** and **4** was comparable to the level of inhibition displayed by TMP.

Compound **2** was bound to *E. coli* DHFR in a manner similar to that observed for TMP by X-ray crystallography. A 2.8 Å crystal structure of the *E. coli* DHFR–**2** complex was solved and showed that the pyrimidine ring of inhibitor **2** was bound to the enzyme in a fashion analogous to that of TMP, as shown in Figure 5. As discussed above and illustrated in Figure 2, TMP is protonated in its complex with DHFR. The X-ray structure of compound **2** bound to DHFR suggested that **2** also was protonated (based on its juxtaposition to the carboxylate of Asp-27) and donated a hydrogen bond from its 2-amino group to a buried water molecule, as observed in the corresponding TMP–enzyme complex. As shown in Figure 2, the 4-amino group of TMP donates hydrogen bonds to the backbone carbonyl groups of Ile-5 and Ile-94. However, compound **2** can donate only one hydrogen bond and, interestingly, appeared to participate in a bifurcated hydrogen bond with its hydrogen atom equidistant from the carbonyl oxygens of the isoleucines 5 and 94. Such a hydrogen-bonding pattern is made possible, in part, by the relatively large bond angle of the pyrrole nitrogen atom with respect to the pyrimidine ring in **2** (angle N7–C7a–N1 = 125° versus angle N(4-amino)–C4–N3 = 117° in TMP[30]). As shown in Figure 5, the trimethoxyphenyl group of inhibitor **2** was similar in position within the active site to the corresponding group of TMP, although bond angle differences at the trigonal 5-carbon of the pyrrolopyrimidine **2** (bond angle of 130°) and the methylene group of TMP (bond angle of 118°) caused the trimethoxyphenyl group of **2** to orient closer to the left side of the active site cleft than that ring of TMP. At the resolution of crystallographic determination of the DHFR–compound **2** complex, no significant differences in protein structure were observed between it and the DHFR complex with TMP.

The basicity of these inhibitors is an important factor in the many effects that determine DHFR binding affinity. As demonstrated for TMP (pK_a of 7.1)[31] and for the anticancer drug methotrexate (pK_a 5.3),[32] the protonated form of the inhibitors binds to the enzyme.[9,13] The measured pK_a of compound **2** was 5.8, indicating that at neutral pH only 6% of the compound is protonated.[33] In contrast, 56% of TMP would be in the protonated state at pH 7.0, at which the I_{50} for *E.*

Table 1. DHFR inhibition data for compounds **1–4**

Compd	DHFR I_{50} (10^{-8} M)				
	E. coli	*S. aureus*	*P. berghei*	*N. gonorrhoeae*	*Rat liver*
1 (TMP)	0.7	4.7	24	45	35000
2	44	3.9	41	18	2900
3	1700	nd[a]	4400	nd	38000
4	14000	nd	nd	>16000	>20000

[a]Not determined.

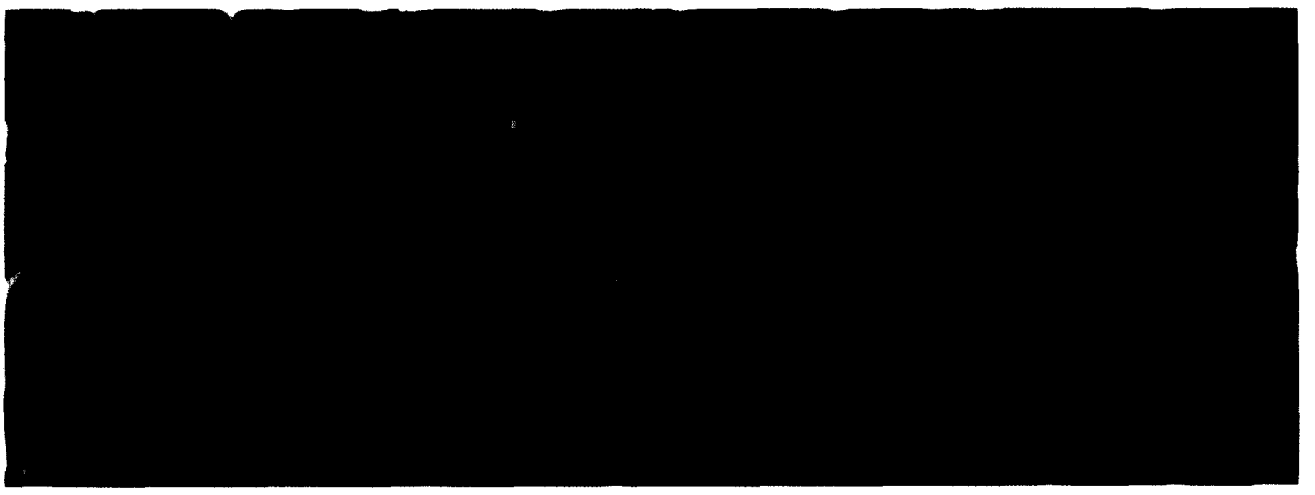


Figure 5. Stereo view of the X-ray crystal structure of compound **2** bound to *E. coli* DHFR superimposed with the structure of the TMP–*E. coli* DHFR complex. Protein structure is represented as a gold tube with atoms of selected active site residues shown. Atoms are color-coded by atom type: carbon, grey; nitrogen, cyan; oxygen, red. Compound **2** is colored teal. The protein structure from the TMP complex is omitted for clarity. The two structures were superimposed by least squares superposition of selected active site alpha-carbons.

coli DHFR inhibition was determined. Therefore, of the 63-fold difference in *E. coli* DHFR binding between compound **2** and TMP, nine-fold could be attributed to the difference in concentration of the protonated species under the neutral pH conditions of the enzyme assay. Thus the intrinsic affinity of the protonated form of compound **2** for *E. coli* DHFR was seven-fold less than that of TMP. Corresponding analyses of the inhibition data for the *S. aureus*, *P. berghei*, *N. gonorrhoeae*, and rat liver enzymes, which were obtained at pH 6.4, 7.3, 6.4, and 7.0, respectively, indicated that the protonated form of **2** exhibited 5-, 8-, 10-, and 110-fold greater inhibitory activity than TMP for these respective proteins. Thus, the structural differences between compound **2** and TMP, which had a relatively small, negative impact on binding of **2** to the *E. coli* enzyme, imparted as much as an order of magnitude of increased binding of the compound to the other bacterial DHFRs and enhanced binding for the mammalian enzyme by two orders of magnitude.

An analysis of the structural differences between TMP and compounds **3** and **4** suggested that the weak DHFR affinity of compounds **3** and **4** was associated to some degree with the orientation of the trimethoxyphenyl group relative to the pyrimidine ring. In both cases the torsion angle about the bond corresponding to C4-C5-CH_2-aryl of TMP was significantly different from that of TMP in its *E. coli* DHFR complex. That torsion angle for TMP was 177°, and the corresponding values from molecular mechanics models of compounds **3** and **4** were 133° and 148°, respectively. (Torsion angles for the enantiomeric partners were 227° and 212°, respectively.) The value of that angle in compound **2** was 180° and closely matched that of TMP. Heterocycle pK_a was not expected to be a significant factor in the weak enzyme binding of compounds **3** and **4**; the basicity of both compounds should be similar to that of TMP. The 4-amino-to-methylene group linker in compounds **3** and **4** may be partly responsible for the low enzyme affinity, but molecular models of those compounds in complex with *E. coli* DHFR did not suggest any particular detrimental interactions between those atoms of the inhibitors and the protein.

One expected effect of conformational restriction was enhanced enzyme selectivity. However, for compound **2**, the difference between the affinity for the bacterial and mammalian enzymes was significantly less than that for TMP, due to its higher affinity for the mammalian protein. The initial expectation of higher selectivity was based on the observed difference in conformational preference of TMP in the two species of enzyme. More recent X-ray crystallographic results of a TMP complex with mouse DHFR[7] provide some insight into the reduced selectivity of compound **2**. The observed conformation of TMP in that complex is essentially identical to that of TMP bound to *E. coli* DHFR, suggesting that the two observed conformations of the drug are isoenergetic within the mouse DHFR active site.

Conclusions

NMR studies of TMP bound to bacterial DHFR indicate a substantial degree of flexibility of the drug within the enzyme active site,[14] but results from the conformationally restricted analogues reported here suggest that relatively precise matching of the TMP conformation observed in the crystal structure of the drug–enzyme complex is necessary for high enzyme affinity. Compound **2**, the best structural mimic of TMP in its complex with bacterial DHFR among the three analogues reported here, showed inhibition essentially equivalent to that of TMP of DHFR from several species of bacteria, and the protonated form of compound **2** showed inhibitory activity of those enzymes that was up to 10-fold greater than that of TMP. X-ray crystallographic analysis of compound **2** bound to *E. coli* DHFR confirmed the expected mode of binding and demonstrated the close similarity of compound **2** with the bacterial enzyme-bound form of TMP.

Experimental

Chemistry

Melting points were determined in open capillaries on a Thomas–Hoover apparatus and are uncorrected. NMR spectra were recorded on a Varian XL-100 or CFT-20 spectrometer. Chemical shifts are in parts per million (δ), relative to the observed solvent resonance (DMSO, 2.50). Analytical samples of compounds moved as single spots on TLC, which were performed on Whatman MK6F silica gel plates. Column chromatography was carried out on silica gel 60 (E. Merck, Darmstadt, Germany). Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA. Electron impact mass spectra were obtained with a Varian MAT CH5-DF mass spectrometer. The pK_a of compound **2** was determined using ultraviolet methods as described for related compounds.[31]

(3,4,5-Trimethoxyphenyl)acetaldehyde (2-amino-6-methyl-4-pyrimidinyl)hydrazone (7). 2-Amino-4-hydrazino-6-methylpyrimidine[23] (3.20 g, 23 mmol) was dissolved in 60 mL of boiling water, and 5.28 g (25 mmol) of 3,4,5-trimethoxyphenylacetaldehyde[24] was added in one portion. The mixture was kept at reflux under nitrogen for 2 h and stored at rt overnight. The resulting yellow solid was collected by filtration and recrystallized from ethanol:water (1:1) to give 5.10 g (67%) of compound **7** as a yellow solid: mp 192–197 °C; Anal. calcd for ($C_{17}H_{23}N_5O_3$): C, 57.99; H, 6.39; N, 21.13; found: C, 57.92; H, 6.43; N, 21.12; ^{1}H NMR (DMSO-d_6): δ 2.11 (s, 3H), 3.49 (d, J=6 Hz, 2H), 3.64 (s, 3H), 3.76 (s, 6H), 5.96 (br s, 2H), 6.16 (s, 1H), 6.54 (s, 2H), 7.40 (t, J=6 Hz, 1H), 10.31 (s, 1H).

2-Amino-4-methyl-5-(3,4,5-trimethoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (2). A mixture of 5.95 g (18.0 mmol) of hydrazone **7** and 60 mL of tetralin was kept at reflux under nitrogen for 48 h. The

solution was cooled to rt and the resulting solid was isolated by filtration. Recrystallization from absolute ethanol afforded 2.80 g (49%) of **2** as a tan crystalline solid: mp 204–207 °C; Anal. calcd for ($C_{16}H_{18}N_4O_3$): C, 61.14; H, 5.77; N, 17.82; found: C, 61.04, H, 5.80; N, 17.80; ^{1}H NMR (DMSO-d_6): δ 2.64 (s, 3H), 3.69 (s, 3H), 3.81 (s, 6H), 6.01 (br s, 2H), 6.70 (s, 2H), 7.00 (d, J = 2 Hz, 1H), 11.2 (br s, 1H).

Ethyl 2-ethoxy-(3,4,5-trimethoxyphenyl)-1-pyrroline-3-carboxylate (11). To a solution of 17.41 g (53.8 mmol) of ethyl 2-oxo-4-(3,4,5-trimethoxyphenyl)-3-pyrrolidine carboxylate (**10**)[25] in 75 mL of dry CH_2Cl_2 was added 16.9 g (88.9 mmol) of triethyloxonium tetrafluoroborate and the solution was stirred at rt for 19.5 h. Ice-cold aqueous sodium carbonate (11.0 g in 150 mL) was added and the mixture was shaken. The organic layer was dried over magnesium sulfate. Solvent was removed on a rotary evaporator to leave a brown oil, which was subjected to column chromatography on silica gel, eluting with diethyl ether:hexanes (3:1). Fractions containing only desired product, as indicated by TLC (silica gel, CH_2Cl_2:methanol, 10:1), were combined and dried over magnesium sulfate. Removal of solvent on a rotary evaporator left 14.2 g (75% yield) of compound **11** as a yellow oil: Anal. calcd for ($C_{18}H_{25}NO_6$): C, 61.52; H, 7.17; N, 3.99; found: C, 61.10; H, 7.20; N, 3.82; ^{1}H NMR ($CDCl_3$): δ 1.28 (t, J = 7 Hz, 3H), 1.33 (t, J = 7 Hz, 3H), 3.7 (m, 1H), 3.82 (s, 3H), 3.84 (s, 6H), 4.0–4.4 (m, 4H), 4.28 (q, J = 7 Hz, 2H), 6.42 (s, 2H).

2-Amino-5-(3,4,5-trimethoxyphenyl)-3,5,6,7-tetrahydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one (12). To a solution of 10.73 g (30.5 mmol) of compound **11** in 80 mL of absolute ethanol was added 8.15 g (72.6 mmol) of potassium *t*-butoxide and 3.25 g (34.0 mmol) of guanidine hydrochloride. The resulting milky-white solution was heated at reflux for 5 h and then evapd to dryness under reduced pressure. The residue was taken up in water and the solution was acidified (pH 2) with 1 N HCl. The precipitate was collected by filtration and washed with ether to furnish 4.07 g of a white solid. A second crop of 1.64 g of white solid was collected for a total yield of 59%. The first crop was recrystallized from methanol to give 1.58 g of compound **12** as a white solid: mp 260–266 °C (dec); Anal. calcd for ($C_{15}H_{18}N_4O_4 \cdot 0.75H_2O$): C, 54.29; H, 5.92; N, 16.88; found: C, 54.39; H, 6.02; N, 16.69; ^{1}H NMR (DMSO-d_6): δ 3.16 (dd, J = 10 and 5 Hz, 1H), 3.62 (s, 3H), 3.71 (s, 6H), 3.78 (t, J = 10 Hz, 1H), 4.15 (dd, J = 10 and 5 Hz, 1H), 6.34 (br s, 2H), 6.43 (br s, 1H), 6.48 (s, 2H), 9.69 (br s, 1H).

2-Amino-6,7-dihydro-5-(3,4,5-trimethoxyphenyl)-5*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl methanesulfonate (13). A solution of 500 mg (1.57 mmol) of compound **12** in about 20 mL dry pyridine was cooled to −42 °C and 0.15 mL (0.22 g, 1.9 mmol) methanesulfonyl chloride was added. The solution was slowly warmed to rt, solvent was removed on a rotary evaporator, and the red oily solid was subjected to flash chromatography on silica gel, eluting with 4% methanol:CH_2Cl_2. Fractions containing desired product, as indicated by TLC (silica gel, CH_2Cl_2:CH_3OH, 10:1), were pooled and evapd to dryness to leave an off-white solid. Washing with $CHCl_3$ (25 mL) left 300 mg (48%) of compound **13** as a white solid: mp 183–186 °C; Anal. calcd for ($C_{16}H_{20}N_4O_6S$): C, 48.48; H, 5.08; N, 14.13; found: C, 48.29; H, 5.16; N, 14.06; ^{1}H NMR (DMSO-d_6): δ 3.37 (dd, J = 10 and 6 Hz, 1H), 3.52 (s, 3H), 3.62 (s, 3H), 3.73 (s, 6H), 3.93 (t, J = 10 Hz, 1H), 4.39 (dd, J = 10 and 6 Hz, 1H), 6.48 (s, 2H), 6.55 (br s, 2H), 7.43 (br s, 1H); MS: $[M]^+$ (396, 88%), $[M-MeSO_2]$ (317, 100%).

2-Amino-5,6-dihydro-5-(3,4,5-trimethoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (3). Mesylate **13** (1.00 g, 2.52 mmol) was subjected to hydrogenation over 0.25 g of 10% Pd on carbon in 20 mL of 50% acetic acid at about 50 °C on a Parr shaker for 18 h. The catalyst was removed by filtration and the solution was washed with five 50 mL portions of CH_2Cl_2. The pH was adjusted to 9.5 with solid NaOH and the resulting white precipitate was isolated by filtration. Recrystallization from water gave 20 mg of compound **3** as a white solid: mp 228–230 °C; Anal. calcd for ($C_{15}H_{18}N_4O_3$): C, 59.59; H, 6.00; N, 18.53; found: C, 59.55; H, 6.05; N, 18.53; ^{1}H NMR (DMSO-d_6): δ 3.3 (m, 1H), 3.64 (s, 3H), 3.74 (s, 6H), 3.85 (m, 1H), 4.33 (m, 1H), 5.86 (br s, 2H), 6.52 (s, 2H), 6.87 (br s, 1H), 7.37 (s, 1H); MS: $[M]^+$ (302, 100%).

(1-(3,4,5-Trimethoxyphenyl)ethylidene)malononitrile (14). A mixture of 100 g (0.476 mol) of 3,4,5-trimethoxyacetophenone, 30 g (0.45 mol) of malononitrile, 22.5 g acetic acid, 7.5 g of ammonium acetate, and about 200 mL of toluene was heated to reflux under a Dean–Stark trap for 1.5 h. The solution was cooled to rt, washed with three 250 mL portions of water, and dried over magnesium sulfate. Removal of solvent on a rotary evaporator left an orange solid, which was washed with absolute ethanol and dried to furnish 71.4 g (58%) of desired product **14** as a yellow solid: mp 120–122 °C; Anal. calcd for ($C_{14}H_{14}N_2O_3$): C, 65.11; H, 5.46; N, 10.85; found: C, 65.18; H, 5.46; N, 10.85; ^{1}H NMR (DMSO-d_6): δ 2.65 (s, 3H), 3.76 (s, 3H), 3.84 (s, 6H), 7.06 (s, 2H); MS: $[M]^+$ (258, 100%).

2-(3-Dimethylamino-1-(3,4,5-trimethoxyphenyl)allylidene) malononitrile (15). A solution of 75 g (0.29 mol) of compound **14** in about 250 mL acetic anhydride was cooled to 0 °C and 40 mL (36 g, 0.30 mol) of dimethyl formamide dimethyl acetal was added dropwise over a period of 15 min. The dark solution was stirred at rt for 2.5 h and diluted with an equal vol of diethyl ether. The resulting precipitate was isolated by filtration and washed with diethyl ether to afford 48.8 g (54%) of compound **15** as a bright yellow solid: mp 208–210 °C; Anal. calcd for ($C_{17}H_{19}N_3O_3$): C, 65.16; H, 6.11; N, 13.41; found: C, 65.07; H, 6.15; N, 13.40; ^{1}H NMR (DMSO-d_6): δ 3.03 (s, 3H), 3.15 (s, 3H), 3.73 (s, 3H), 3.79 (s, 6H), 5.67 (d, J = 12 Hz, 1H), 6.60 (s, 1H), 7.10 (d, J = 12 Hz, 1H); MS: $[M]^+$ (313, 100%).

2-Bromo-4-(3,4,5-trimethoxyphenyl)-3-pyridinecarbonitrile (16). To a stirred mixture of 40.0 g (0.128 mol) of compound **15** in about 100 mL of acetic acid was added dropwise 150 mL of 30% HBr/acetic acid. The reaction was monitored for remaining starting material by TLC (silica gel, CH_2Cl_2) during the HBr addition, and upon disappearance of **15** the mixture was poured onto ice/sodium carbonate. The resulting solid was isolated by filtration and taken up in a CH_2Cl_2-extract of the filtrate. This solution was passed over a pad of silica gel and evapd to dryness to leave a pale yellow solid. The solid was washed with ether and dried to furnish 36 g (80%) of **16** as a white solid: mp 147–149 °C; Anal. calcd for ($C_{15}H_{13}N_2O_3Br$): C, 51.60; H, 3.75; N, 8.02; found: C, 51.56; H, 3.67; N, 8.00; ^{1}H NMR (DMSO-d_6) δ 3.75 (s, 3H), 3.85 (s, 6H), 7.05 (s, 2H), 7.79 (d, J=5 Hz, 1H), 8.66 (d, J=5 Hz, 1H); MS: M^+ (348, 100%; 350, 98%).

2,4-Diamino-5-(3,4,5-trimethoxyphenyl)pyrido[2,3-*d*]pyrimidine (17). To a mixture of 20.0 g (57.0 mmol) of compound **16** and 20.25 g (212 mmol) of guanidine hydrochloride in 250 mL of *t*-butanol was added cautiously 8.93 g (212 mmol) of 57% NaH in mineral oil. The grey mixture was stirred at rt for 1 h and heated at reflux for 2.5 h. TLC (silica gel, CH_2Cl_2:CH_3OH, 4:1) analysis of the mixture indicated that the reaction was not complete, and 5.45 g (57.1 mmol) of guanidine hydrochloride and 2.42 g (57.5 mmol) of 57% NaH/mineral oil were added. The mixture was heated at reflux for 1 h and filtered hot. The filter cake was washed with two 50 mL portions of hot *t*-butanol, two 50 mL portions of water, and two 50 mL portions of acetone. The remaining off-white solid was dried at 100 °C to leave 12.6 g (68%) of crude product, a sample of which was recrystallized from water to give analytically pure **17**: mp > 270 °C; Anal. calcd for ($C_{16}H_{17}N_5O_3$): C, 58.71; H, 5.23; N, 21.40; found: C, 58.64; H, 5.30; N, 21.02; ^{1}H NMR (TFA-*d*): δ 4.01 (s, 6H), 4.15 (s, 3H), 6.88 (s, 2H), 7.68 (d, J=6 Hz, 1H), 8.92 (d, J=6 Hz, 1H); MS: M^+ (327, 100%).

2-Amino-5,6,7,8-tetrahydro-5-(3,4,5-trimethoxyphenyl)pyrido[2,3-*d*]pyrimidin-4-one (19). A solution of 10.0 g (42.2 mmol) of compound **17**, 250 mL of 1 N NaOH, and 100 mL of 2-methoxyethanol was heated at reflux for 28 h. The solution was cooled to rt and filtered, and the filtrate was neutralized with concd HCl. The resulting white precipitate was collected by filtration and washed with hot methanol to leave 9.6 g of impure **18** as a white gummy solid. Catalytic hydrogenation of compound **18** using platinum oxide (1.0 g) in 70 mL of trifluoroacetic acid was performed in a Parr hydrogenator and was complete in 45 min. Catalyst was removed by filtration through a pad of Celite, and the filtrate was concd to a vol of about 10 mL on a rotary evaporator and neutralized with 100 mL of aq satd sodium carbonate. The resulting grey precipitate was isolated by filtration and washed with water. The solid was recrystallized from ethanol, using activated carbon for decolorization, to give 2.98 g (20% based on **17**) of compound **19** as a white solid: mp 153–157 °C; Anal. calcd for ($C_{16}H_{20}N_4O_4 \cdot 0.8H_2O$): C, 55.42; H, 6.28; N, 16.16; found: C, 55.42; H, 6.31; N, 16.16; ^{1}H NMR (DMSO-d_6): δ 1.76 (m, 2H), 2.95 (m, 2H), 3.61 (s, 3H), 3.70 (s, 6H), 3.89 (m, 1H), 6.02 (br s, 2H), 6.36 (s, 2H), 6.41 (s, 1H), 9.82 (br s, 1H); MS: M^+ (332, 98%).

2-Amino-5,6,7,8-tetrahydro-4-methylsulfonyloxy-5-(3,4,5-trimethoxyphenyl)pyrido[2,3-*d*]pyrimidine (20). A solution of 2.50 g (7.52 mmol) of compound **19** in 50 mL of 2,6-lutidine was cooled to 0 °C and 0.8 mL (1.2 g, 10 mmol) of methanesulfonyl chloride was added. The solution was allowed to warm to rt and stirred for a total of 1.5 h. TLC (silica gel, CH_2Cl_2:CH_3OH, 10:1) indicated that the reaction was incomplete, and an additional 0.8 mL of methanesulfonyl chloride was added. The solution was concentrated on a rotary evaporator and diluted with water. The cloudy solution was kept at 4 °C overnight to produce a yellow oil. Trituration with ethanol gave an off-white solid. The aqueous solution was concentrated and cooled to afford a white solid. The two batches of product were washed with water and dried to yield a total of 2.24 g (73%) of compound **20** as an off-white solid: mp 174–175 °C; Anal. calcd for ($C_{17}H_{22}N_4O_6S$): C, 49.75; H, 5.40; N, 13.65; found: C, 49.69; H, 5.43; N, 13.64; ^{1}H NMR (DMSO-d_6): δ 1.89 (m, 2H), 2.88 (m, 2H), 3.55 (s, 3H), 3.63 (s, 3H), 3.70 (s, 3H), 4.02 (m, 1H), 6.25 (br s, 2H), 6.32 (s, 2H), 7.29 (br d, J=4 Hz, 1H); MS: M^+ (410, 27%), [M − $MeSO_2$] (331, 25%).

2-Amino-5,6,7,8-tetrahydro-5-(3,4,5-trimethoxyphenyl)pyrido[2,3-*d*]pyrimidine (4). A solution of 1.50 g (3.65 mmol) of compound **20** was subjected to hydrogenation over 0.5 g of 5% Pd/C in 25 mL of 50% acetic acid at 60 °C on a Parr hydrogenator for 18 h. Catalyst was removed by filtration through a pad of Celite and the filtrate was washed with two 50 mL portions of methylene chloride. The aq soln was basified with NaOH pellets and then 5 N NaOH to a pH of about 10 to produce a white precipitate, which was isolated by filtration. The solid was recrystallized from ethanol to give 128 mg (11%) of compound **4** as a white crystalline solid: mp 220–221 °C; Anal. calcd for ($C_{16}H_{20}N_4O_3$): C, 60.75; H, 6.37; N, 17.71; found: C, 60.67; H, 6.41; N, 17.68; ^{1}H NMR (DMSO-d_6): δ 1.91 (m, 2H), 3.20 (m, 2H), 3.64 (s, 3H), 3.72 (s, 6H), 3.83 (m, 1H), 5.66 (br s, 2H), 6.43 (s, 2H), 6.83 (br s, 1H), 7.14 (s, 1H); MS: $[M]^+$ (316, 100%); UV (0.1 N HCl): λ_{max} 276 nm (ε 13,800), λ_{min} 263 nm (ε 12,800).

Enzyme isolation and assays

Assays for *E. coli*, *N. gonorrhoeae*, *P. berghei*, and rat liver DHFR were performed at pH 7.0, 6.4, 7.3, and 7.0, respectively, as previously described.[34]

S. aureus (strain S8862) was grown in Brain Heart Infusion Broth at 37 °C to late log phase (A_{600}=1–1.5). The cells were harvested by centrifugation and resuspended in 50 mM Tris–HCl (pH 8), 24% NaCl. Lysostaphin (10 mg) was added to the 50 mL suspension, and the mixture was stirred for 1 h at rt then centri-

fuged. The pellet was lysed by the addition of 50 mM Tris–HCl (pH 8). DNase, RNase, and $MgCl_2$ were added to the suspension. After homogenization and chilling to 5 °C, another centrifugation was performed, and the supernatant was fractionated by precipitation between 40 and 90% satd $(NH_4)_2SO_4$. The precipitate was dissolved in 50 mM Tris–HCl (pH 8), 1 mM DTT, 1 mM EDTA and applied to an Ultrogel AcA-54 gel filtration column equilibrated with 50 mM potassium phosphate buffer (pH 6.9), 0.1 M KCl, 1 mM DTT, 1 mM EDTA. DHFR activity eluted in the 18,000–20,000 mol wt range, and the pooled enzyme (0.3 U/mg) was dialyzed against 50 mM potassium phosphate buffer (pH 6.9), 1 mM DTT, 1 mM EDTA, and was free of interfering NADPH oxidase activity.

Kinetic analyses of *S. aureus* DHFR were performed in 0.1 M imidazole Cl pH 6.4. The apparent K_m for NADPH (9.2 mM) was determined in the presence of 45 mM dihydrofolate, and the apparent K_m for dihydrofolate (5.4 mM) was determined in the presence of 60 mM NADPH. In both cases the mercaptoethanol concentration was kept at 12 mM. I_{50} values were determined from plots of percentage of inhibition vs the logarithm of inhibitor concentration.

X-ray Crystallography

Crystals of *E. coli* DHFR in binary complex with compound **2** were grown by co-crystallization of the compound with protein as previously described for the TMP complex.[8] Crystals were space group $P6_1$, with $a = 93.6$ Å, $c = 73.9$ Å having two independent binary complexes per asymmetric unit. A total of 7549 unique reflections were collected to about 2.8 Å resolution having Rsymm ($\Sigma|F_i - \langle F \rangle|/\Sigma F_i|$) of 0.089. A set of unrefined coordinates derived from earlier molecular modeling, and based on the coordinates of DHFR in binary complex with TMP, were refined using the program PROFFT.[29] The ligand molecule was remodeled with SYBYL software, and its geometric parameters were generated by the program GEN_IDEALS.[35] Several rounds of refinement were done starting with 3.5 Å data and finally with data extending to 2.8 Å. A total of 7234 reflections (over 1 * sigma) were included, representing about 83% of the possible unique data. The model, comprising 2536 protein atoms, 46 ligand atoms, and 208 solvent atoms, had rms deviations from ideal bond lengths, planarity, and peptide torsion angles of 0.014 Å, 0.005 Å and 4.8°, respectively. A difference Fourier map, plotted at the 2 * sigma density level and examined on an Evans and Sutherland PS300 workstation with FRODO software,[36] showed no significant difference features in the active site region, indicating modeling to be essentially complete.

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