Brief Articles

Crystal Structure of the Hydrated Strontium Salt of Methotrexate: Two Independent Molecules with Different Conformations§

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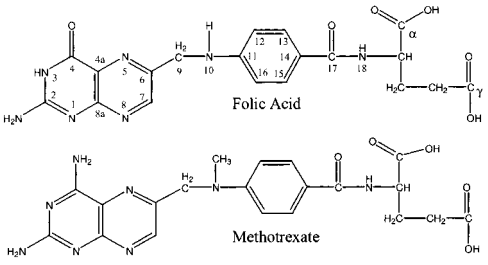
The crystal and molecular structure of methotrexate has been determined by X-ray diffraction from a highly hydrated triclinic crystal form in which the asymmetric unit contains two independent methotrexate molecules with their glutamate carboxyl groups coordinated to two strontium ions. The two methotrexates exhibit differing conformations: They are almost related to one another by a pseudocenter of symmetry. This places the C(9)−N(10) bond vectors on opposite sides of the planes of the pteridine rings. The 2,4-diaminopteridines form 2-fold symmetry-related hydrogen-bonded dimers as well as hydrogen bonds to benzoyl carbonyl oxygens and lattice water molecules. This structure provides experimental proof of the existence of pteridine conformers through rotation about the C(6)−C(9) bond. Comparison of these conformers with other free and enzyme-bound methotrexate conformations shows them all to be different and illustrates the ability of the molecule to adapt to its chemical environment. The results from this crystal structure determination are experimental proof that methotrexate has not one preferred molecular conformation but may freely rotate about several bonds. They also suggest that the dihydrofolate reductase-bound methotrexate conformation is greatly influenced by the specific binding site environment of the enzyme.

Introduction

The enzyme system dihydrofolate reductase−thymidylate synthetase (DHFR−TS), which is essential for the synthesis of DNA components, continues to be a major target area in the search for effective anticancer drugs. If we could design DHFR or TS inhibitors which would be more efficiently taken up by cancer cells or which would have affinity for drug-resistant and/or mutated cancer cells, they would block tumor DNA production selectively and thus be valuable chemotherapeutic agents. Since folate derivatives are both substrates for DHFR and cofactors for TS, folic acid has long been a model compound for the design of DHFR and TS inhibitors for use as potential anticancer drugs. Methotrexate (MTX), which differs from folic acid only by replacement of a carbonyl oxygen atom by an amino group and substitution of a methyl group for a hydrogen atom, is a very powerful DHFR inhibitor and a widely used cancer chemotherapy drug.

Rational development of new antifolate anticancer drugs would be helped by identification of the molecular reasons for MTX's strong DHFR inhibition. Some have been revealed by crystal structure analyses of tetrahydrofolates[1] and of folic acid[2] and comparisons with the structure of MTX bound to DHFR[3] and by an NMR



investigation of hydride transfer in the DHFR reduction of folic acid.[4] These studies have independently shown that despite the close chemical similarity between folate substrates and 2,4-diaminofolate inhibitors (MTX, aminopterin) of DHFR, the inhibitors bind to the enzyme with the pteridine ring rotated approximately 180° from the pteridine orientation of the enzyme-bound substrates (for a review see ref 5). The pteridine ring orientation in the crystal structure of folic acid was shown[2] to be stabilized through a water-mediated intramolecular hydrogen bond between the N(10) hydrogen and C(4) carbonyl oxygen. This placed the ring in an orientation similar to what was found in DHFR−folate crystal structures.[6] Two isomorphous crystal structures of MTX reported to date[7,8] have determined a conformation for the pteridine ring that is different from that observed in the DHFR−MTX complex structures[3] and more like the DHFR−folate structures. Therefore the question still remains as to whether the pteridine orientation in DHFR-bound MTX is a preferred or stabilized conformation or is induced through free rotation in binding to DHFR. As the rational design

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of new DHFR inhibitors as potential anticancer drugs would substantially benefit from resolution of this question, we have investigated crystallization of MTX under a variety of conditions. We report the crystal and molecular structure of MTX in a crystal form in which the asymmetric unit consists of two independent MTX molecules, two strontium ions, and several water molecules. The two MTX molecules display different pteridine orientations thus providing an experimental demonstration of the accessibility of two conformers through rotation of these rings. The structure determination also enables us to compare additional free MTX molecular conformations with that of enzyme-bound MTX and with folic acid.

Experimental Section

Crystals of the strontium salt of MTX were grown from a refrigerated (5 °C) ethanol/water solution at pH 7.5 by very slow evaporation. The crystals cracked on standing in air and consequently a single crystal measuring 0.36 × 0.29 × 0.05 mm was sealed in a capillary tube with mother liquor present for collection of X-ray data. X-ray photographs and diffraction measurements indicated a primitive triclinic lattice with unit cell dimensions: $a = 8.4435(6)$, $b = 10.6655(7)$, $c = 19.199(1)$ Å; $\alpha = 92.251(5)$, $\beta = 98.868(5)$, $\gamma = 92.881(5)°$. Density considerations suggested the unit cell to consist of 2 strontium ions and 2 MTX molecules plus about 25 water molecules. Since the MTX sample used contained only the L-glutamyl isomer, the space group was assumed to be *P*1. X-ray intensity data, collected on a four-circle diffractometer to a maximum $2\theta = 120°$ with Ni-filtered copper radiation, totaled 5023 independent reflections, of which 4373 had $I > 2\sigma(I)$. During data collection, the crystal tended to pick up a small amount of mother liquor, which caused it to shift slightly in the capillary tube. After each movement, data collection was stopped and the crystal was recentered. Due to the significant absorption of Cu radiation by strontium (the calculated compound linear absorption coefficient = 36.83 cm^{-1}) an absorption correction was necessary. Crystal movement in the capillary plus a varying amount of solvent accumulation around the crystal precluded the application of an empirical correction based on rotation about a scattering vector. As an alternative, an analytical absorption correction[10] was applied which took into account the crystal dimensions and the orientation matrix that was obtained after each recentering of the crystal.

The structure was solved by the heavy atom method. Positions for the two strontium ions were determined from a sharpened Patterson map, and the remaining atoms were obtained from subsequent difference Fourier maps. Interpretation of the maps was difficult in the early stages due to the false center of symmetry introduced by the heavy atoms. Eventually 2 MTX molecules were located along with 20 other maxima which were assigned to water oxygens. Another 11 peaks,which appeared in the difference maps, were assigned to disordered water oxygens and given partial occupancy factors ranging from 0.25−0.67. Hydrogen atoms for the geometrically fixed positions were calculated for both MTX molecules and were included as well as hydrogen atom positions located on electron density difference maps for the methyl groups and the ordered water molecules bringing the total number of atom positions in the asymmetric unit to 179.

Full-matrix least-squares refinement of 99 non-hydrogen atom positions using anisotropic temperature factors for the strontium ions and MTX molecules and isotropic factors for all water oxygens yielded a discrepancy index *R* of 0.076 and a weighted *R* of 0.084 for the observed data. Hydrogen parameters were not refined. It should be pointed out that limits in the accuracy of the diffraction data, reflected in the crystallographic *R* value, most likely are due to crystal movement, the accuracy of the applied absorption correction, and disorder of a number of water molecules found in the lattice.

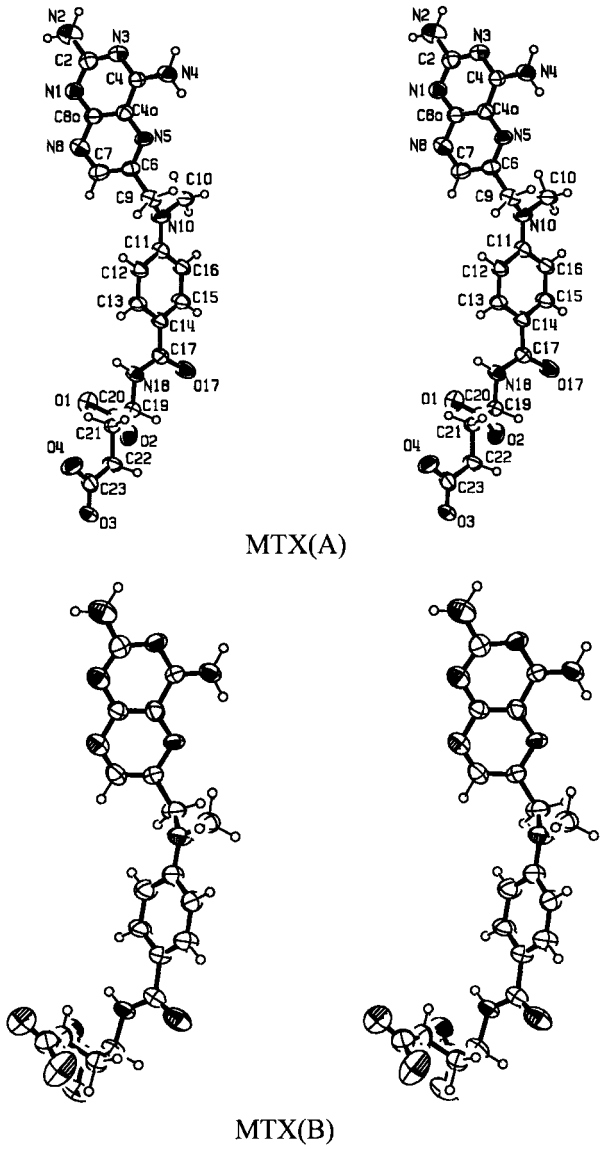

Figure 1. Stereoviews of MTX molecule A with chemical numbering scheme (top) and MTX B (bottom).

Results and Discussion

Figure 1 shows a perspective drawing of the two MTX molecules viewed with their pteridine rings in similar orientations. Figure 2 illustrates the packing of the two independent MTX molecules, the strontium ions, and the water molecules in the crystal unit cell. The pteridine and the *p*-aminobenzyol groups are clustered about halfway up the *c* crystal axis, and the glutamate carboxylic groups project above and below this more hydrophobic region forming a hydrophilic area with the water molecules and strontium ions. Figure 3 shows just the coordination geometry about the strontium ions. Each strontium ion (Sr(1), Sr(2)) is coordinated to carboxyl oxygen atoms of both MTX molecules (MTX-(A), MTX(B)) and to several water molecules. Sr(1) makes short contacts to the γ-carboxylate oxygen of MTX(A) (2.54 Å), the α-carboxylate oxygen of a translationally related MTX(A) (2.48 Å), and the two γ-carboxylate oxygens of MTX(B) (2.64, 2.63 Å). Sr(1) is also

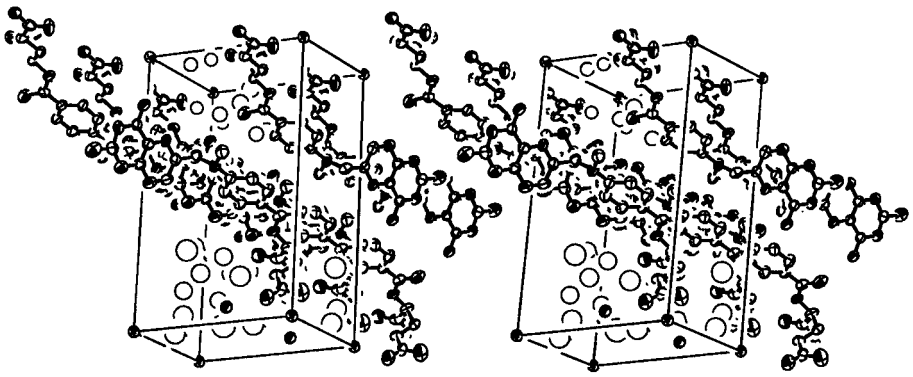

Figure 2. Stereoview of the unit cell of the strontium salt of MTX. Water oxygens are represented as unfilled circles.

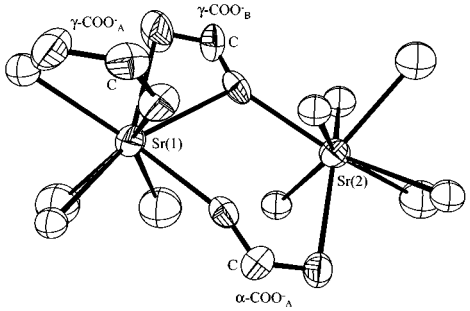

Figure 3. Coordination geometry about the strontium ions.

coordinated to four water molecules with Sr(1)−O(water) distances ranging from 2.62 to 2.71 Å, which brings the total coordination number of Sr(1) to 8. Sr-(2) is coordinated to the other α-carboxylate oxygen of MTX(A) (2.66 Å) and to one of the γ-carboxylate oxygens of MTX(B) (2.54 Å) which forms a bridge between Sr(1) and Sr(2). Sr(2) is coordinated to six waters with Sr(2)−O(water) distances ranging from 2.64 to 2.69 Å; thus the coordination number about Sr(2) is also equal to 8. The α-carboxylate group of MTX(B) does not take part in coordination to the strontium ions directly, but its one oxygen is hydrogen-bonded to two water molecules that are coordinated to Sr(2). The other oxygen is hydrogen-bonded to one other water molecule (2.78 Å).

Except for the C(23)−O(4) distances, all other equivalent bond lengths in the two independent MTX molecules agree to within 3σ. The C(23)−O(4) distances are 1.28(2) Å in MTX(A) and 1.18(2) Å in MTX(B), which are clearly significantly different but are within the range for normal C═O bonds. In the tetrahydrate structure[7] the C(2)−N(2) and C(4)−N(4) distances were found to be 1.281(8) and 1.305(7) Å, respectively, whose average bond length (1.29(2) Å) is less than the average length of the eight pteridine C−N endocyclic bonds (1.34(2) Å). It is believed that with the N(1) of the 2,4-diaminopteridine protonated in the tetrahydrate structure, the tendency toward shorter C−N amino bonds is a result of increased double-bond character in these bonds. In the MTX(A) and MTX(B) molecules the average length of the 4 C−N amino bonds is 1.36(4) Å, while the average of the 16 C−N endocyclic bond lengths is 1.35(3) Å. Thus with the N(1)'s of MTX(A) and MTX-(B) not protonated, the endocyclic and exocyclic pteridine C−N bond lengths are more equal and there is less double-bond character in the amino groups.

The two MTX molecules are aligned in the unit cell such that they are almost related by a noncrystallographic center of symmetry that passes through the point (0.44, 0.30, 0.57). The apparent center of symmetry relationship is of course broken by the L-glutamic acid moieties. However the orientations of the pteridine and *p*-aminobenzoyl segments of the two molecules are almost exactly related by the pseudocenter of symmetry. This relationship is described quantitatively in Table 1 by a comparison of selected torsion angles in the two MTX molecules and is clearly illustrated in Figure 5 which shows the two independent molecules in their contrasting alignments. An important feature of this pseudocentrosymmetric relationship is the differing orientations of the 2,4-diaminopteridine ring in the two

Table 1. Selected Torsion Angles (deg) for Unbound Folic Acid and Unbound and Enzyme-Bound MTX

	atoms defining torsion angle			
	N(5)−C(6)−C(9)−N(10)	C(6)−C(9)−N(10)−C(11)	C(9)−N(10)−C(11)−C(12)	C(13)−C(14)−C(17)−N(18)
folic acid[a]	31	180	−13	−4
MTX(A)[b]	−111	−141	30	15
MTX(B)	113	136	−29	−13
MTX-tetrahydrate[c]	42	62	19	−6
H-DHFR[d]	−150	65	3	−24
L. casei[e]	−157	57	5	−14
E. coli(A)[e]	−162	55	5	−36
E. coli(B)[e]	−157	51	15	−32

[a] Ref 2. [b] This work. [c] Refs 7−8. [d] MTX bound to human DHFR (ref 14), coordinates obtained from the Protein Data Bank. [e] MTX bound to *L. casei* and *E. coli* DHFRs (ref 3), coordinates obtained from the Protein Data Bank.

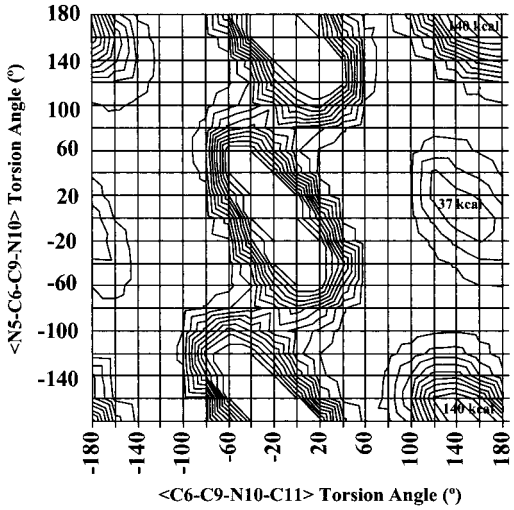

Figure 4. Two-dimensional plot of the variation in the calculated energy with change in the N(5)−C(6)−C(9)−N(10) and C(6)−C(9)−N(10)−C(11) torsion angles for MTX.

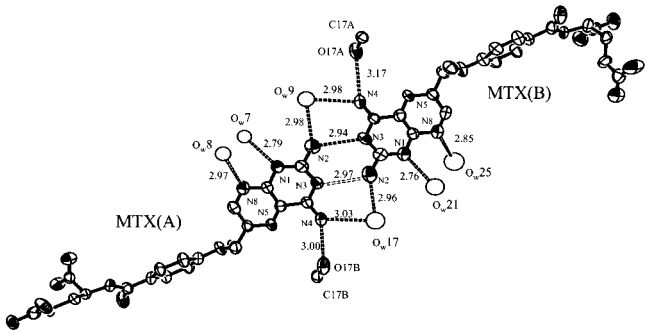

Figure 5. View of the hydrogen-bonded dimer interaction between the MTX(A) and MTX(B) pteridine rings including hydrogen bonds to water oxygens (O_w) and benzoyl carbonyl groups. Distances are given in Å.

molecules. As shown in Table 1, the torsion angles which describe the relationship of the pteridine ring to the rest of the molecule are opposite in sign from one MTX to the other. The C(9)−N(10) bond vectors are on opposite sides of the pteridine rings and nearly perpendicular to the ring planes. A similar perpendicular arrangement was found in the reported structure of a quinazoline antifol.[9] The present crystal structure determination provides experimental evidence that the MTX molecule does not prefer one conformation only but may rotate about many single bonds including those linking the pteridine ring to the rest of the molecule. The specific observed MTX conformations in this crystal form may of course be influenced to some extent by crystal packing forces. However, we emphasize that two differing conformations in the same crystal provide direct experimental proof that such distinctly different orientations of the pteridine ring and other parts of the MTX molecule are indeed physically possible and that rotations about most of the single bonds within the MTX molecule (to achieve the two observed conformations) are allowed.

To further investigate the rotational flexibility of the MTX molecule, we have done a simple mechanics calculation[11] (van der Waals + electrostatic energy) to obtain a two-dimensional energy profile about the N(5)−C(6)−C(9)−N(10) and C(6)−C(9)−N(10)−C(11) torsion angles. Using MTX(A) as the model, we allowed minor adjustments about the N(10)−C(10) bond to minimize any unfavorable contacts between the methyl group hydrogens and neighboring atoms. The energy profile results of the C(6)−C(9) and C(9)−N(10) bond rotations are shown in Figure 4. The plot is contoured at 10-kcal intervals, and the contouring was truncated at the 100-kcal level. There is an obvious restricted region that occurs with the C(9)−N(10) torsion angle set between −100° and +60°. The highest barriers in this region (1310 and 1270 kcal/mol) which occur with the C(6)−C(9) torsion angle around 0° and 180° are the result of a close contact between the C(7)−H and a benzene hydrogen. The next highest barriers are caused by the close approach of the N(10) methyl group with the C(7)−H (140 kcal/mol) and with the N(4) amino group and N(5) atom (37 kcal/mol). It is evident that when the C(6)−C(9)−N(10)−C(11) torsion angle is approximately 70° or −120° there is no significant energy barrier to 360° rotation about the C(6)−C(9) bond (maximum energy barrier in this region is 7 kcal/mol). Similarly when the N(5)−C(6)−C(9)−N(10) torsion angle is in the range 70−100° or (−70)−(−100)°, there is no significant barrier to rotation greater than 180° about the C(9)−N(10) bond (rotating in the positive direction from 60° to −100°, the maximum energy barrier is 5 kcal/mol in this region). These results illustrate the correlation between the C(6)−C(9) and C(9)−C(10) bond torsion angles in determining the observed conformations of MTX. It is evident that rotation of the pteridine ring with no significant energy barrier may be accomplished through appropriate alterations in adjoining atom torsion angles. We also note from Table 1 that for unbound folic acid and the Sr-MTX structures, torsion angles of 180° and 140°, respectively, about the C(9)−N(10) bond places the *p*-aminobenzoyl-L-glutamate (pABG) group in an extended conformation away from the pteridine ring. In the enzyme complexes as well as in the MTX-tetrahydrate structure, the pABG is bent so as to place the N(10)−C(11) bond vector nearly perpendicular to the plane of the pteridine ring. Incorporating a fixed or preferred bent conformation in the design of new antifolates may help in the development of more potent or selective DHFR inhibitors.

Hydrogen Bonding. The pteridine rings of the two MTX molecules in the asymmetric unit are linked in the crystal by hydrogen bonds (Figure 5). There are direct links between the N(3) atom and N(2) amino group of each molecule forming a near-2-fold symmetry-related hydrogen-bonded dimer. In addition, the N(2) amino group of each molecule is indirectly linked to the other molecule's N(4) amino group through hydrogen bonding with an intervening water molecule. Solution[12] and crystal structure studies of DHFR complexed with MTX or folate derivatives have consistently found a water molecule that forms bridging hydrogen bonds between the enzyme and the N(2) amino group of the ligand. The N(4) amino groups also form hydrogen bonds with the *p*-aminobenzoyl carbonyl oxygen of a translationally related MTX molecule of the opposite kind. Similar hydrogen bonds between peptide carbonyl groups and the N(4) amino group are also found in MTX−DHFR complexes. Other hydrogen-bonding interactions of the pteridine rings involve the N(1) and N(8) atoms of both molecules with other water molecules. The pteridine N(5) atoms of both molecules do not participate in any hydrogen bonds in this crystal structure. The glutamate carboxyl oxygens of the two MTX molecules form additional hydrogen bonds (not shown in the figure) with both ordered and partially occupied water molecules in the lattice. Although there are many water molecules in the Sr-MTX lattice, none hydrogen-bonds to two pteridine atoms of the same molecule and there is no intramolecular hydrogen-bonding stabilization of conformation such as was found in the crystal structure of folic acid.

The N···N hydrogen bonding between adjacent molecules is a common feature found in the crystal structures of other 2,4-diamino antifolates.[9,13] This contrasts with the absence of such intermolecular N···N hydrogen bonds found in the MTX-tetrahydrate crystal structure. In that structure crystallization at pH 4 favored protonation of N(1) and concomitant α-carboxyl group ionization resulting in a zwitterion hydrogen-bonded pair. Hydrogen bonding of the pteridine ring to ionizable carboxyl groups of specific amino acids of DHFR, such

as aspartate and glutamate, directly, and mediated by intervening water molecules, is found in the crystal structures of substrate/inhibitor−DHFR complexes and is important for holding the ring in the binding pocket. The MTX-Sr salt structure has no protonated nitrogens but does share some of the hydrogen bonding features with the bound MTX structures as pointed out above.

Conclusions

With the addition of the present crystal structure, four different conformations of MTX have been determined: the enzyme-bound conformation, MTX-tetrahydrate, and the two strontium salt conformers. It is readily apparent that the MTX molecule is indeed flexible and can adapt to its surrounding chemical environment. This crystal structure determination provides direct experimental evidence of the lack of one preferred or stabilized pteridine conformation in MTX and suggests that binding conformation may be largely determined by the architecture of enzyme binding sites. A two-dimensional energy profile calculation supports the claim of free rotational flexibility about the C(6)−C(9) bond provided there are appropriate adjustments in rotation about the C(9)−N(10) bond. Differences in the rotations about the C(6)−C(9) and C(9)−N(10) bonds are particularly important since they control the relative orientations of the pteridine and *p*-aminobenzyol groups which have been observed to prefer a particular folded conformation when bound to DHFR. One of the factors responsible for the tight binding of MTX to DHFR is the excellent hydrogen-bonding capability of the pteridine ring. Intermolecular hydrogen bonding is also observed to be an important feature in the crystal packing of the unbound structures. These results suggest that care must be taken in the design of rigid or fixed conformation MTX analogues. Compounds with restricted rotational freedom in the C(6), C(9), N(10) region may not be effective DHFR inhibitors if the rotational restrictions do not allow adoption of the enzyme-binding conformation. Similarly, substitutions on the pteridine ring that prevent hydrogen bonding will also hinder binding to DHFR.

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Supporting Information Available: Tables of crystal data, atomic coordinates, anisotropic thermal parameters, and bond lengths and angles. This material is available free of charge via the Internet at http://pubs.acs.org.

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