Molecular Structures of 2,4-Diaminopyrimidine Antifolates with Antineoplastic Activity[1]

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2,4-Diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) and its ethanesulfonate salt (DAMP-ES) are potent inhibitors of mammalian dihydrofolate reductase and also inhibit the growth of cultured cells as effectively as the drug methotrexate (MTX). DAMP is currently in phase I clinical studies. An analogue of DAMP having 5-(1-naphthyl) in place of the adamantyl group (DNMP) possesses little cytotoxic as well as enzyme inhibitory activity. The crystal and molecular structures of DAMP-ES and DNMP were determined in order to elucidate the conformational aspects of drug specificity. The molecular conformation of DAMP-ES shows that the C8–C7 bond of the adamantyl ring is nearly coplanar with the pyrimidine ring (C8–C7–C5–C6 = −7.5°) instead of staggered as expected from steric considerations. As a result, the pyrimidine ring and its 4,6-substituents are severely distorted from coplanarity. In DNMP, the 1-naphthalene ring is perpendicular to the pyrimidine ring (C8–C7–C5–C6 = −87.0°) which is itself planar. N1 is protonated in DAMP-ES but not in DNMP. When the two structures are compared, the 5-substituents occupy different regions of space, with the outer ring of the naphthalene group outside of the volume occupied by the adamantyl ring. Therefore, the reduced effectiveness of DNMP may be caused by the inability of the naphthalene to fit the binding site in dihydrofolate reductase. This is the situation when DNMP is placed in the methotrexate binding site of *Lactobacillus casei* crystal structure.

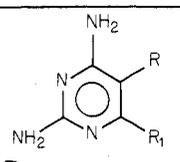
Dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase, EC 1.5.1.3) is a key enzyme involved in the transfer of one-carbon units in the biosynthetic pathways leading to the formation of the purine and pyrimidine nucleotides, the precursors of DNA and RNA. Therefore, inhibition of this enzyme will ultimately lead to cell death. Methotrexate (MTX), although closely related in chemical structure to the natural DHFR substrates, folic acid and dihydrofolic acid, is a potent inhibitor of this enzyme and is clinically the most widely used antifolate chemotherapeutic agent.[2,3] However, numerous studies have shown that the structure of methotrexate may be considerably modified and still retain inhibitory potency. The principal structural characteristics necessary for binding to dihydrofolate reductase appear to be a 2,4-diaminopyrimidine or *S*-triazine structure.[4-8]

Further structure–activity studies on 2,4-diaminopyrimidines show that a lipophilic group at position 5 is essential for tight binding of the compound to DHFR. Comparison of several 5-lipophilic substituted analogues indicate that those with a 5-adamantyl substituent are the most effective inhibitors of dihydrofolate reductase.[9-11] The effect of the 5-adamantyl group appears, to a certain extent, dependent on its hydrophobicity, since it has been shown that within similar structures (Table I) the affinity for dihydrofolate reductase increases with increased hydrophobicity.[7] However, there are other unknown, presumably conformational, factors which make apparently similar compounds differ greatly in their affinity for this enzyme,[7,10] in particular, the great variations in binding affinity of the naphthalene analogues and the adamantyl derivatives (Table I).

It was further shown that this class of antifolates is specific toward mammalian DHFR and possess antineoplastic activity,[12,13] whereas methotrexate is not species specific.[5] At present, DAMP (Table I) is entering phase I clinical trials.

Explanations for the high binding affinity of the 2,4-diaminopyrimidine antifolates with respect to the binding of the 2-amino-4-oxofolate substrates have focused on the modified pattern of hydrogen bond donors and acceptors[6] or on a change in the electron density of the pyrimidine ring leading to increased basicity.[4]

Table I. Structure–Activity Relationships



compd	R	R_1	activity: ID_{50},[a] M
	adamantyl	CH_2CH_3	0.00025
DAMP	adamantyl	CH_3	0.006
	adamantyl	H	0.33
	NHCO-adamantyl	CH_3	1.0
	CH_2NHCO-adamantyl	H	3.9
	$CH_2NHCO(CH_2)_8CH_3$	H	1.1
	$(CH_2)_2CH_3$	CH_3	53
	$(CH_2)_5CH_3$	CH_2CH_3	0.34
	cyclohexyl	CH_3	0.13
DNMP	1-naphthalene	CH_3	56
	2-naphthalene	CH_3	0.07
	1-naphthalene	CF_3	
	methotrexate		0.008

[a] ID_{50} is the concentration needed for 50% growth inhibition of mouse mammary adenocarcinoma cells (TA3) in culture.

Therefore, in order to investigate the conformational aspects of the specificity of lipophilic 2,4-diamino-

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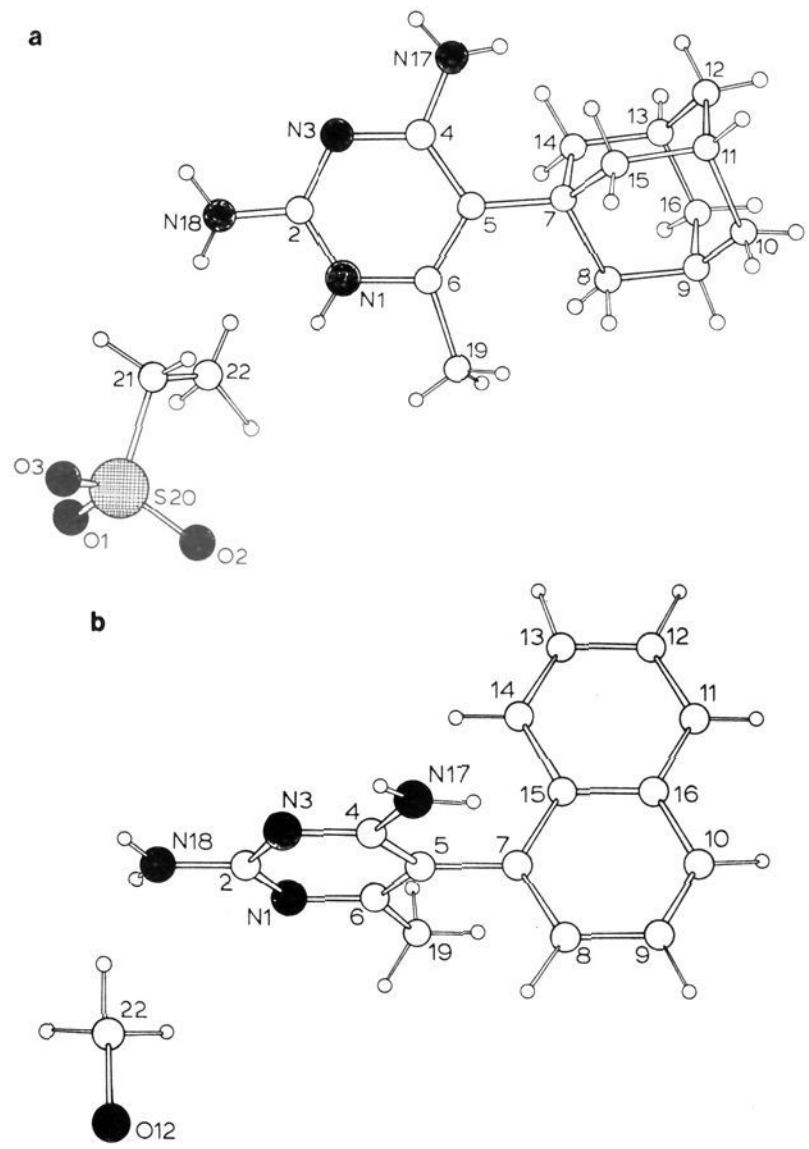

Figure 1. (a) Molecular structure and numbering scheme for 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine ethanesulfonate salt (DAMP-ES). K_i for dihydrofolate reductase is 0.009 μM (competitive inhibitor). (b) Molecular structure and numbering scheme for 2,4-diamino-5-(1-naphthyl)-6-methylpyrimidine–methanol complex (DNMP-M). K_i for dihydrofolate reductase is 56 μM (noncompetitive inhibitor).

pyrimidines for binding to dihydrofolate reductase and to describe the hydrogen-bonding patterns in protonated and nonprotonated pyrimidines, the crystal structures of the active antineoplastic antifolate 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) ethanesulfonate salt and the inactive, noncompetitive analogue 2,4-diamino-5-(1-naphthyl)-6-methylpyrimidine (DNMP)–methanol complex (Figure 1) were studied, and the results are described here.

Experimental Section

Crystals of both DAMP-ES and DNMP-M were grown at room temperature from methanol solutions. Samples were synthesized as previously described.[9,10] The crystal data for both compounds are listed in Table II.

Both structures were solved by application of direct method techniques using MULTAN[14] and refined anisotropically by full-matrix least-squares techniques. Hydrogen atoms were located in difference electron density Fourier maps and refined isotropically for both structures. Positional and anisotropic thermal parameters for all nonhydrogen atoms, positional and isotropic parameters for hydrogen atoms, packing diagrams, and lists of calculated structure factors are available (see paragraph at end of paper regarding supplementary material).

Results

The geometry (Figures 2–4) and molecular conformations (Figure 1) of these two antifolate compounds are

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Table II. Crystal Data for 2,4-Diamino-5-(1-adamantyl)-6-methylpyrimidine Ethanesulfonate and 2,4-Diamino-5-(1-naphthyl)-6-methylpyrimidine–Methanol Complex

	DAMP-ES	DNMP-M
mol formula	$C_{15}H_{22}N_4O_3S$	$C_{16}H_{18}N_4O$
M_r	338.0	282.4
crystal system	triclinic	monoclinic
space group	$P\bar{1}$	$C2/c$
Z	2	8
cell dimensions		
a, Å	10.309 (1)	18.542 (9)
b, Å	14.536 (2)	10.990 (4)
c, Å	6.486 (1)	14.721 (9)
α, deg	91.76 (2)	90.0
β, deg	94.98 (1)	91.905 (9)
γ, deg	109.75 (1)	90.0
vol, Å^3	909.4	2998.1
density (calcd), g/cm^3	1.35	1.26
crystal size, mm	0.16 × 0.16 × 0.80	0.42 × 0.24 × 0.12
λ, Å	1.5418	1.5418
μ, cm^{-1}	17.4	6.6
R, %	5.4; 3387 data	8.0; 1484 data

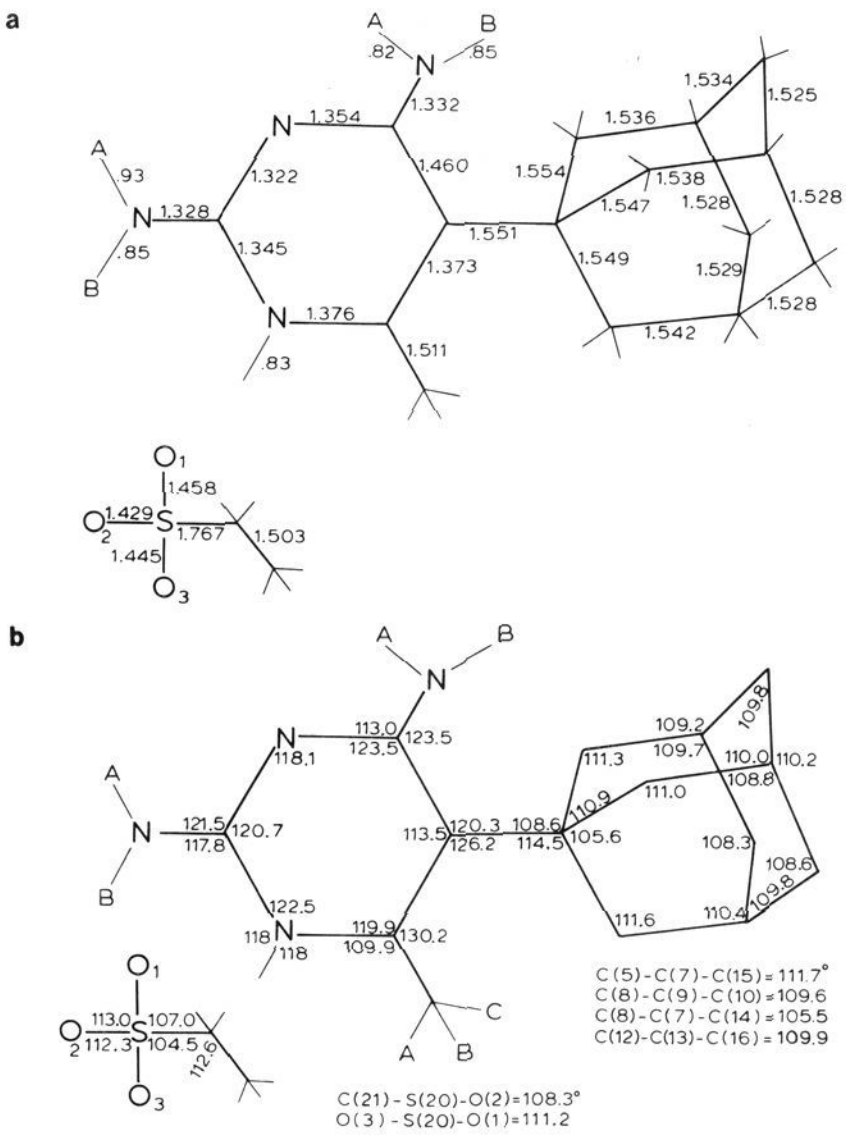

Figure 2. Bond distances (a) and bond angles (b) in DAMP-ES.

described. As illustrated (Figures 1a and 4), the conformation of DAMP is such that the C7–C8 bond of the adamantyl ring is nearly coplanar with the pyrimidine ring (C8–C7–C5–C6 = –7.5°) instead of staggered (≃30°) as might be expected from steric considerations. As a result, there is steric interference of the 6-methyl hydrogens with the adamantyl ring (H19A···H8A = 2.05 Å) as well as the N17 hydrogens (H17B···H15B = 1.88 Å; H17B···H14A = 2.15 Å). In order to relieve these unfavorable interactions, the pyrimidine ring and its 4,6-substituents are severely distorted from coplanarity (Figure 4). The largest distortions are reflected in the twist of the 4- and 6-substituents above and below the pyrimidine plane (N17-C4-

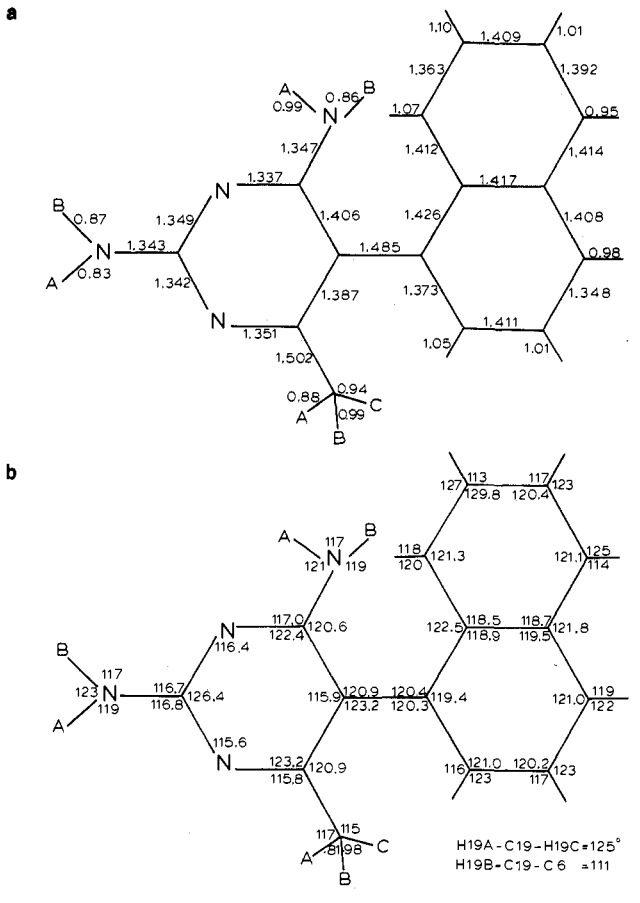

Figure 3. Bond distances (a) and bond angles (b) in DNMP-M.

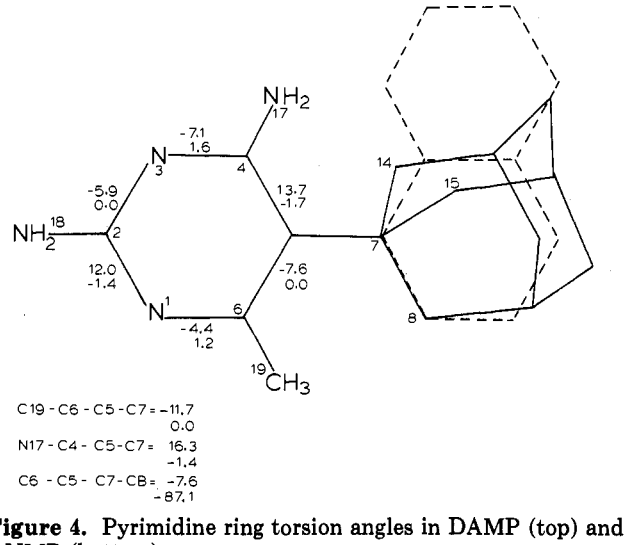

Figure 4. Pyrimidine ring torsion angles in DAMP (top) and DNMP (bottom).

C5-C7 = 16.3°; C19–C6–C5–C7 = −11.7°), respectively. In addition, C7 is 0.36 Å above the pyrimidine plane. To further relieve this steric strain, the angles involving the 6-methyl and 5-adamantyl groups are expanded: C19–C6–C5 = 130.2°, C6–C5–C7 = 126.2°, and C5–C7–C8 = 114.5° (Figure 2b). None of these distortions is evident in DNMP (Figure 3b). The naphthalene ring is perpendicular to the pyrimidine ring (C8–C7–C5–C6 = −87.1°), which is itself planar. The 4,6-substituents of DNMP are also coplanar with the pyrimidine ring.

The disruption of the pyrimidine ring planarity in DAMP is the result of the steric strain caused by the adamantyl ring and is not influenced by the protonation at N1, since other protonated antifolates are coplanar.[15]

Table III. Hydrogen Bonding in DAMP-ES and DNMP-M

D-H···A	D-H, Å	H···A, Å	D···A, Å	<DHA, deg
	DAMP-ES			
N1-H···O3	0.87	1.93	2.77	161
N17-HA···O1	0.89	2.19	2.93	141
N17-HB···N3	0.81	2.35	3.15	170
N18-HA···O1	0.86	2.00	2.90	176
N18-HB···O2	0.85	2.47	3.25	154
	DNMP-M			
N17-HA···N3	0.91	2.89	2.99	170
N17-HB···O1	0.88	2.39	3.12	142
N18-HA···N1	0.84	2.20	3.04	176
O1-H···O1	0.78	2.47	3.06	128

However, protonation at N1 does change the geometry of the pyrimidine ring and its substituents, with the geometry of DAMP and DNMP being indicative of their respective protonation states. The short amino bonds in DAMP (Figure 2a) suggest immonium character and good proton donor capability. The shortened C5–C7 bond in DNMP is indicative of double bond character from π contributions of the naphthalene ring. The adamantyl and naphthyl rings, as well as the solvent molecules, ethanesulfonate and methanol, all show normal geometry.

The hydrogen-bonding patterns of these two structures (Table III) show that all the amino hydrogens of DAMP-ES and all but one of DNMP-M participate in the hydrogen bonding. This is in contrast to that observed in the structures of trimethoprim[16] and diveridine,[17] which as neutral, nonsolvated structures employ only one of each amino hydrogen in the hydrogen-bonding scheme.

Discussion

2,4-Diaminopyrimidines with lipophilic groups attached to position 5 possess antifolate activity as demonstrated by their cytotoxic activity for cultured cells, which can be reversed by hypoxanthine, thymidine, and glycine, and by their inhibition of dihydrofolate reductase. In particular, those with adamantyl are very strong cytotoxic agents and, in this respect, resemble the drug methotrexate.[12] It appears also that those pyrimidines with rigid substituents at C-5 have a higher affinity for dihydrofolate reductase than those with hydrocarbon chains of comparable molar volume and hydrophobicity.[7] DAMP-ES is presently entering phase I clinical study. On the other hand, the 5-(1-naphthyl) derivative shows no competitive binding or cytotoxic activity, even though it has similar lipophilicity. Thus, it is of interest to investigate the structural characteristics of the adamantyl and naphthyl derivatives (Figure 1).

When the molecular volumes[18] of DAMP and DNMP are compared (Figure 5) it is observed that the 5-substituents occupy different regions of space, with the volume of the naphthyl ring beyond that occupied by the adamantyl ring. Therefore, it is possible that the reduced effectiveness of DNMP relative to DAMP is the inability

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(18) Molecular volumes are computed using a pseudoelectron density function computed on the MMS-X graphics display system, designed and fabricated by the Computer Systems Laboratory, Washington University, St. Louis. This system was made possible by a grant (RR-00396) from the Division of Research Resources, NIH, under their Biotechnology Resource Program.

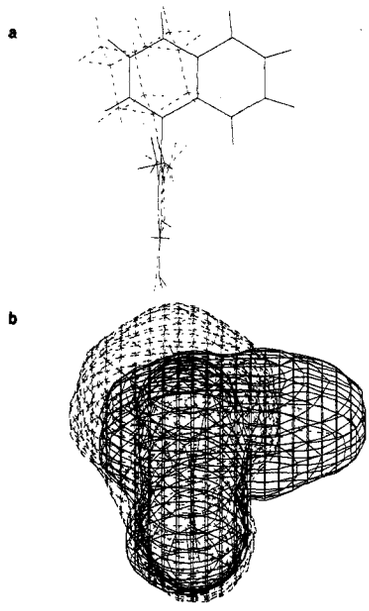

Figure 5. (a) Superposition of DAMP and DNMP, viewed parallel to the pyrimidine ring showing the distortions of DAMP from coplanarity and the extended volume occupied by the naphthalene ring in DNMP. (b) Superposition of the molecular volumes of DAMP (dashed) and DNMP (solid), generated on the MMS-X graphics, viewed in the same orientations as 5a.

of the naphthalene ring to effectively occupy the active site of dihydrofolate reductase.

The crystallographic studies of methotrexate complexes with dihydrofolate reductase from *E. coli*[19] and *L. casei*[20] show that the methotrexate binding environment is preserved in both structures. Methotrexate is protonated at N1 in both, and it hydrogen bonds to the carboxylic oxygens of an aspartic acid; the 2-amine hydrogen bonds to the hydroxyl of a threonine, while the 4-amine hydrogen bonds to the carbonyl oxygen of an isoleucine. Since both DAMP and DNMP have the same 2,4-diamino ring system, they could also participate in the same hydrogen-bonding scheme. The hydrogen bonding of DAMP-ES (Table III), as a protonated species, exhibits the same characteristics as the methotrexate–DHFR complexes, with all the nitrogen functions binding to oxygen functional groups.

To investigate the possible binding environment of DAMP and DNMP when bound to dihydrofolate reductase, we carried out computer modeling studies using the coordinates of the *L. casei* DHFR–MTX–NADPH ternary complex.[20,21] In each case, the 2,4-diaminopyrimidine ring was placed in the same orientation observed for the methotrexate diamino groups in the ternary complex. When DAMP (Figure 6a) is inserted in the MTX site, the adamantyl ring occupies a hydrophobic pocket. There are no unusually short intermolecular contacts to the adamantyl

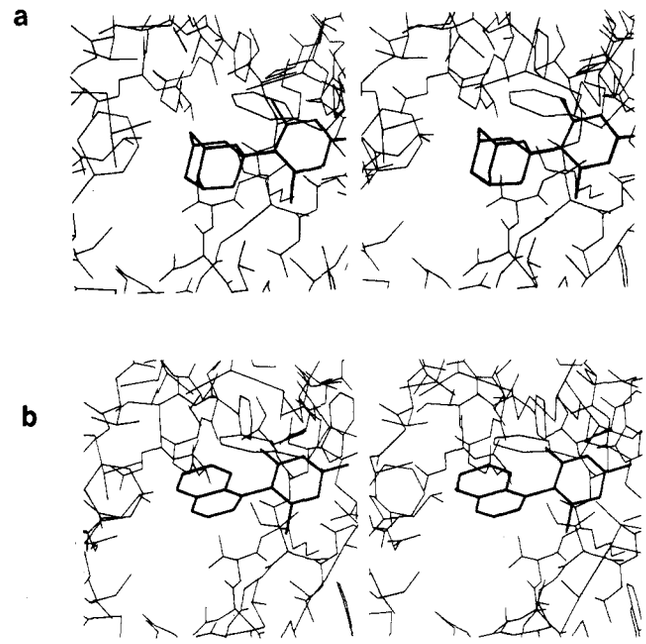

Figure 6. Methotrexate binding site of *L. casei* dihydrofolate reductase ternary complex[20] with (a) DAMP replacing methotrexate and (b) DNMP replacing methotrexate. The NADPH has been removed for clarity.

ring, although the NADPH may not be present when DAMP binds, since it is involved in the catalytic site of dihydrofolate reductase. However, the situation encountered for DNMP binding (Figure 6b) is dramatically different. In its observed conformation, there are severe steric interactions between the naphthalene ring and the side chains lining the binding pocket. While many of the most severe interactions are relieved when the naphthalene ring is rotated about the C5–C7 bond, there is no single orientation that is satisfactory. However, it must be noted that these antifolates are specific to mammalian reductases, and the details of the binding interactions may not be conserved.[22]

Therefore, these structural results suggest that the great differences observed in the DHFR binding affinity (Table I) for DAMP and DNMP with similar lipophilicities are due to the steric bulk of the naphthalene ring not being easily accomodated by the reductase active site.

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Supplementary Material Available: Positional and anisotropic thermal parameters for all nonhydrogen atoms, positional and isotropic parameters for hydrogen atoms, packing diagrams, lists of calculated structure factor amplitudes and stereoviews of the crystal packing in the DAMP-ES and DNMP-M lattices (32 pages). Ordering information is given on any current masthead page.

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(21) The molecular modeling of DAMP and DNMP was kindly provided by Dr. David Matthews, La Jolla, CA.

(22) **Note in Added in Proof:** Since submission of this paper, the binary complex of chicken liver DHFR and DAMP has been determined by D. Matthews. There are small changes in the tertiary structure when DAMP binds to the enzyme–coenzyme complex.