Further Evaluation of 5-Alkyl-5-deaza Antifolates. 5-Propyl- and 5-Butyl-5-deaza Analogues of Aminopterin and Methotrexate

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5-Propyl-5-deaza and 5-butyl-5-deaza analogues of classical antifolates were synthesized by extensions of a previously reported general route which proceeds through 2,4-diamino-5-alkylpyrido[2,3-*d*]pyrimidine-6-carbonitrile intermediates followed by reductive condensation with diethyl *N*-4-(aminobenzoyl)-L-glutamate to give diethyl esters of 5-alkyl-5-deazaaminopterin types. N^{10}-Methyl derivatives, *i.e.*, derivatives of 5-alkyl-5-deazamethotrexate, were also prepared by reductive methylation of the N^{10}-H compounds. 5-Ethyl-5-deazamethotrexate was prepared using an alternative route through 6-(bromomethyl)-2,4-diamino-5-ethylpyrido[2,3-*d*]pyrimidine. These antifolates were evaluated for inhibition of dihydrofolate reductase (DHFR) from L1210 cells, their effect on L1210 and S180 tumor cell growth in culture, and carrier-mediated transport through L1210 cell membranes. Inhibitory effect on DHFR was lowered relative to methotrexate in 5-propyl-5-deazaaminopterin and 5-propyl-5-deazamethotrexate by 2- to 3-fold (K_i = 9.3 and 11.7 p*M*, respectively, *vs.* 4.3 p*M* for methotrexate) and by 17- to 18-fold in 5-butyl-5-deazaaminopterin and 5-butyl-5-deazamethotrexate (K_i = 74 and 78 p*M*, respectively). Molecular modeling using graphics derived from human DHFR show the propyl and butyl compounds interacting with the enzyme in conformations that account for these slight decreases in binding.

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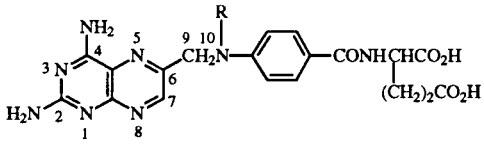
Aminopterin and methotrexate (structures shown below) are established antitumor agents whose cytotoxicity stems from potent inhibition of dihydrofolate reductase (DHFR, 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3). Without production of tetrahydrofolate by DHFR, cells cannot carry out biosyntheses of nucleotide precursors of nucleic acids [1].

Aminopterin was synthesized before methotrexate and was undergoing clinical trials against leukemia when methotrexate was introduced [2]. Methotrexate and aminopterin are equally potent inhibitors of DHFR, but investigators soon discovered methotrexate to be the more therapeutic agent. The rapid uptake and retention of methotrexate by leukemic cells in contrast to normal intestinal epithelial cells largely account for its greater therapeutic index over aminopterin [2-4]. This observation served as impetus in quests for methotrexate analogues of greater antifolate selectivity for tumor over normal proliferative tissue.

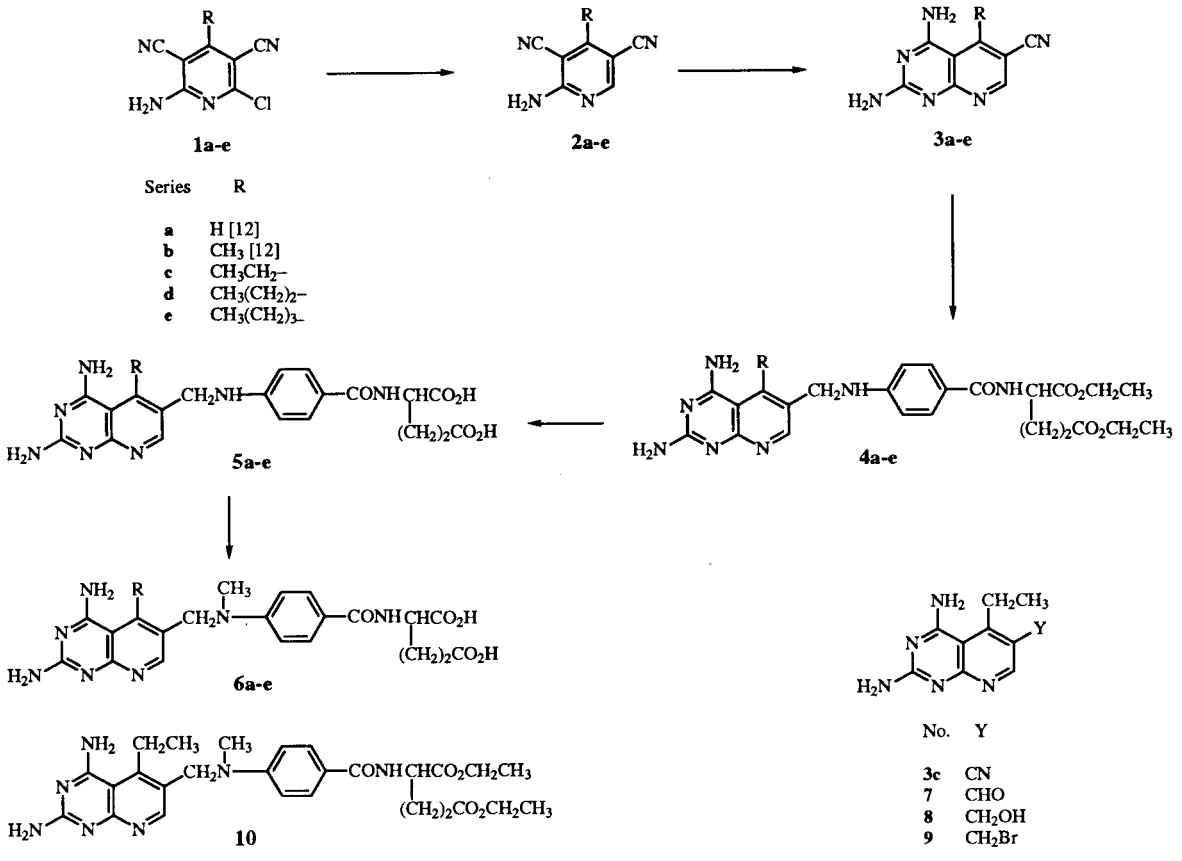


Aminopterin: R = H
Methotrexate: R = CH_3

Many investigators have contributed to the present understanding of essential structural features for antitumor activity by classical antifolates, that is, aminopterin/methotrexate types [5]. One of the means of studying such features has been the replacement of nitrogen by carbon to create various deaza analogues [6]. Studies from our Laboratories have shown that modifications in the 5-and 10-positions may lead to compounds of antitumor activity greater than that of methotrexate [4], and the most favorable effects have been observed in 5- and 10-deaza analogues, particularly in those bearing alkyl substituents at positions 5 and 10. In an earlier paper we reported the high levels of activity of the 5-methyl and 5-ethyl derivatives of 5-deazaaminopterin and 5-deazamethotrexate against several tumor models [7]. In the 10-deaza series, 10-ethyl-10-deazaaminopterin (10-EDAM, edatrexate) was first reported in 1974 [8] and was soon shown to be superior in efficacy and selectivity over methotrexate in many tumor models [9,10]. These findings prompted us to synthesize 5-methyl-10-ethyl-5,10-dideazaaminopterin, an analogue in which the structural features of therapeutically advantageous 5- and 10-deaza types are combined; but, surprisingly, this compound proved to be less active than methotrexate [11].

In the work reported in this paper we extended our previous studies on 5-methyl- and 5-ethyl-5-deaza types [7,12] to 5-propyl and 5-butyl homologs (see Scheme 1) with the aim of evaluating the effects of the bulkier alkyl substituents on antitumor properties. Another part of the present report involves further studies of the use of computer-aided drug design based on the three-dimensional structure of human DHFR [12] as a means to design new inhibitors of this enzyme.

Scheme 1



Chemistry.

The synthetic transformations are outlined in Scheme 1. Syntheses of compounds of the **a** series (R = hydrogen) and the **b** series (R = methyl) were reported previously [12]. Antitumor data on the target compounds of the **c** series (R = ethyl) have been reported [7], but their syntheses have not heretofore been described.

2-Amino-4-alkyl-6-chloropyridine-3,5-dicarbonitriles **1c-e** were subjected to palladium-promoted hydrogenolysis to give 2-amino-4-alkylpyridine-3,5-dicarbonitriles **2c-e**. In the next step, treatment of the **2** types with guanidine to give 2,4-diamino-5-alkyl-5-deazapteridine-6-carbonitriles (structural type **3**), the bulky alkyl groups had the expected steric hindrance effects in progressive fashion. We earlier reported the tlc-monitored reactions of **2a** and **2b** with guanidine in refluxing ethanol; 5-unsubstituted **3a** was obtained in 95% yield after 24 hours whereas 5-methyl compound **3b** was obtained in 58% yield after a 5-day reflux period [12]. When the same conditions were applied in treatment of **2c** with guanidine, the conversion to **3c** was only slight (<5%) after a prolonged reaction period of greater than a week. More stringent conditions were needed in order to convert **2c-e** to **3c-e**. A satisfactory procedure was realized through use of reaction temperatures of 150-160° in 2-(2-methoxyethoxy)ethanol. Yields of 20-23% were obtained after careful purifications using silica gel column chromatography (see Table 1). Reductive condensations in acetic acid (promoted by Raney Ni) of **3c-e** with diethyl *N*-(4-aminobenzoyl)-L-glutamate afforded the diethyl esters **4c-e** from which the target 5-alkyl-5-deazaaminopterin compounds **5c-e** were obtained following ester hydrolysis. Both the condensation and ester hydrolysis procedures were similar to those used to prepare the lower homologs [12]. The ester precursors **4c-e** were carefully purified by column chromatography on silica gel before hydrolysis to the target compounds. Yields of the pure esters were typically in the 15% range, although we had one exceptional preparation in which the yield of **4c** was 32%. The size of the alkyl group at position 5 did not appear to influence the desired conversion; the yields of **4a** and **4b** reported earlier were also 15%. The N^{10}-methyl derivatives **6c-e**, 5-alkyl-5-deazamethotrexate types, were prepared from **5c-e** by reductive methylation (formaldehyde and sodium cyanoborohydride) as described earlier for **6b** [12].

5-Ethyl-5-deazamethotrexate (**6c**) was also prepared by

Table 1

Melting Points, Yields and Analytical Data for Compounds of Scheme 1 [a]

Compound No.	Yield, %	Mp, dec	Molecular Formula [b]	Analyses Calcd./Found %C	%H	%N
1c	27	224-227 [c]	$C_9H_7ClN_4$	52.31	3.41	27.11
				52.19	3.35	27.23
1d	28	222-226	$C_{10}H_9ClN_4$	54.43	4.11	25.39
				54.45	4.00	25.41
1e	27	206-207	$C_{11}H_{11}ClN_4$	56.30	4.72	23.87
				56.29	4.84	24.02
2c	81	176-177	$C_9H_8N_4$	62.77	4.68	32.54
				62.72	4.85	32.38
2d	91	164-166	$C_{10}H_{10}N_4$	64.50	5.41	30.01
				64.46	5.67	29.81
2e	90	142-143	$C_{11}H_{12}N_4$	65.98	6.04	27.98
				66.02	6.01	28.03
3c	20	310-312	$C_{10}H_{10}N_6 \cdot 0.25H_2O$	54.91	4.84	38.42
				55.22	4.60	38.10
3d	23	288-290	$C_{11}H_{12}N_6 \cdot 0.15H_2O$	57.21	5.37	36.39
				57.31	5.41	36.14
3e	20	258-260	$C_{12}H_{14}N_6 \cdot 0.3H_2O$	58.19	5.94	33.93
				58.33	5.82	33.55
4c	32	218-220	$C_{26}H_{33}N_7O_5 \cdot 0.4H_2O$	58.83	6.42	18.47
				58.87	6.54	18.41
4d	16	231-233	$C_{27}H_{35}N_7O_5 \cdot 0.5H_2O$	59.33	6.64	17.94
				59.24	6.56	17.68
4e	14	233-235	$C_{28}H_{37}N_7O_5 \cdot 0.75H_2O$	59.51	6.87	17.35
				59.56	6.81	17.58
5c	85	—	$C_{22}H_{25}N_7O_5 \cdot 1.5H_2O$	53.43	5.71	19.83
				53.47	5.73	19.83
5d	69	—	$C_{23}H_{27}N_7O_5 \cdot 1.75H_2O$	53.85	5.99	19.11
				53.81	5.99	19.11
5e	51	—	$C_{24}H_{29}N_7O_5 \cdot 1.5H_2O$	55.17	6.17	18.76
				55.12	6.03	18.59
6c	66 [d]	—	$C_{23}H_{27}N_7O_5 \cdot 1.7H_2O$	53.94	5.98	19.14
				53.97	5.92	19.05
6d	59	—	$C_{24}H_{29}N_7O_5 \cdot 1.7H_2O$	54.78	6.21	18.63
				54.54	6.46	18.74
6e	29	—	$C_{25}H_{31}N_7O_5 \cdot 2.75H_2O$	53.71	6.58	17.54
				53.87	6.37	17.70

[a] Compounds of series **a** and **b** were reported earlier [12]. [b] Each entry produced a mass spectrum in agreement with the assigned structure. [c] Lit [21] mp 219-221°. [d] From **5c** by reductive alkylation; prepared also from diethyl ester **10** (see Experimental).

an alternative route. The versatile intermediate 6-(bromomethyl)-2,4-diamino-5-ethylpyrido[2,3-*d*]pyrimidine (**9**) was prepared from **3c** in three steps outlined in Scheme 1. Alkylation of diethyl *N*-[(4-methylamino)benzoyl]-L-glutamate with **9** afforded diethyl ester **10**, which was hydrolyzed to give **6c** identical with the sample obtained by reductive methylation of **5c**.

Biological Test Results.

The target compounds were evaluated as folate antagonists with respect to the following properties: (a) their inhibition of DHFR isolated from L1210 cells, (b) their inhibitory effect against the growth of L1210 and S180 cells in culture, and (c) their inward flux into L1210 cells. Results are shown in Table 3. Aminopterin, methotrexate and previously reported **5a**, **5b**, **6a**, and **6b** are included in the listing for purposes of comparison. Antifolate and antitumor evaluations, both *in vitro* and *in vivo*, on the 5-unsubstituted-5-deaza and the lower 5-alkyl-5-deaza homologs were presented earlier [7].

Results in Table 3 show that analogues **5a-c** and **6a-c** exert the same potent inhibition of DHFR as their parent compounds aminopterin or methotrexate. 5-Propyl compounds **5d** and **6d** show slight decreases in inhibitory potency; their effect is lowered by 2- to 3-fold relative to the parent compounds. The 5-butyl analogues **5e** and **6e** show a greater decrease with their inhibitory effect lowered 18- to 20-fold less than the parent compounds.

As inhibitors of the growth of L1210 cells in culture, each of the N^{10}-H compounds **5d** and **5e** exert about the

Table 2

Proton NMR Spectral Data on Target Compounds **5c-e** and **6c-e**

Compound No.	^{1}H NMR (DMSO-d_6), δ relative to TMS
5c	1.20 (t, CH_3CH_2) 1.93, 2.02 (two m, overlapping, -CHCH_2CH_2-, non equivalent), 2.32 (t, CH_2CO), 3.06 (q, CH_3CH_2), 4.3 (two m, overlapping, NHC*H*CH_2 and CH_2N*H*), 6.50 (m, overlapping signals, CH_2N*H* and NH_2), 6.66 and 7.68 (two d, C_6H_4), 7.18 (br s, NH_2), 8.06 (d, CONH), 8.54 (s, C^7-H).
5d	0.92 (t, $CH_3(CH_2)_2$-), 1.58 (m, $CH_3CH_2CH_2$-), 1.96, 2.02 (two m, -CHCH_2CH_2-, non equivalent), 2.32 (t, CH_2CO), 3.04 (t, $CH_3CH_2CH_2$), 4.3 (two m, overlapping, NHC*H*CH_2 and CH_2N*H*), 6.54 (m, overlapping signals, CH_2N*H* and NH_2), 6.66 and 7.68 (two d, C_6H_4), 7.16 (br s, NH_2), 8.07 (d, CONH), 8.54 (s, C^7-H).
5e	0.85 (t, $CH_3(CH_2)_3$-), 1.35 (m, $CH_3CH_2(CH_2)_2$-), 1.52 (m, 2, $CH_3CH_2CH_2CH_2$), 1.96, 2.02 (2m, -CHCH_2CH_2, non equivalent), 2.32 (t, CH_2CO), 3.06 (t, $CH_3(CH_2)_2CH_2$-), 4.3 (two m, overlapping, NHC*H*CH_2 and CH_2N*H*), 6.54 (t, CH_2N*H*), 6.66 and 7.66 (two d, C_6H_4), 6.88 (br s, NH_2). 7.32 (br s, NH_2), 8.08 (d, CONH), 8.54 (s, C^7-H).
6c	1.17 (t, CH_3CH_2), 1.94, 2.02 (two m, overlapping, $CHCH_2CH_2$, non equivalent), 2.30 (t, CH_2CO), 3.0 (m, CH_3CH_2, overlapping with s due CH_3N), 4.33 (q, NHC*H*CH_2), 4.70 (s, CH_2N), 6.76 and 7.75 (two d, C_6H_4), 6.82 (br s, NH_2), 7.32 (br s, NH_2), 8.13 (d, CONH), 8.19 (s, C^7-H).
6d	0.90 (t, $CH_3(CH_2)_2$), 1.54 (m, $CH_3CH_2CH_2$), 1.95, 2.03 (two m, $CHCH_2CH_2$, non equivalent), 2.32 (t, CH_2CO), 3.0 (m, $CH_3CH_2CH_2$, overlapping with s due to CH_3N), 4.34 (q, -NHC*H*CH_2-), 4.68 (s, CH_2N), 6.58 (br s, NH_2), 7.18 (br s, NH_2), 6.77 and 7.75 (two d, C_6H_4), 8.16 (d, CONH), 8.20 (s, C^7-H).
6e	0.85, (t, $CH_3(CH_2)_3$-), 1.31 (m, $CH_3CH_2(CH_2)_2$-), 1.48 (m, $CH_3CH_2CH_2CH_2$), 1.93, 2.02 (two m, $CHCH_2CH_2$, non equivalent), 2.32 (t, CH_2CO), 3.0 (m, $CH_3CH_2CH_2CH_2$ overlapping with s due to CH_3N), 4.34 (q, NHC*H*CH_2), 4.68 (s, CH_2N), 6.62 (br s, NH_2), 6.76 and 7.74 (two d, C_6H_4), 7.20 (br s, NH_2), 8.15 (d, CONH), 8.20 (s, C^7-H).

Table 3

Summary of Data from Biochemical and Growth Inhibition Studies with Aminopterin (AM), Methotrexate (MTX), and 5-Deaza Analogues **5a-e** and **6a-e** [a] [b] [c]

Compound No.	Abbreviated trivial name [d]	L1210 cell DHFR inhibiton K_i (p*M*)	Cell growth inhibition (*vs.* L1210 and S180) IC_{50} (n*M*) L1210	S180	L1210 cell influx K_i (μ*M*)
—	AM	3.55 ±0.4	0.72 ±0.1	1.0 ±0.3	1.2 ±0.3
5a	5-DAM [a]	3.65 ±0.7	0.63 ±0.07	3.7 ±0.4	1.1 ±0.2
5b	5-Me-5-DAM [a]	2.93 ±0.1	0.13 ±0.02	0.57 ±0.2	1.2 ±0.2
5c	5-Et-5-DAM	5.54 ±0.6	0.22 ±0.03	0.26 ±0.3	1.1 ±0.2
5d	5-Pr-5-DAM	9.34 ±1	0.88 ±0.2	4.5 ±0.1	0.65 ±0.1
5e	5-Bu-5-DAM	74.3 ±0.7	0.78 ±0.1	4.5 ±1.7	0.58 ±0.1
—	MTX	4.28 ±0.8	2.55 ±0.3	7.9 ±3.1	4.1 ±0.6
6a	5-DMTX [a]	5.26 ±0.7	2.85 ±0.3	5.7 ±0.7	4.0 ± 0.5
6b	5-Me-5-DMTX [a]	2.12 ±0.4	0.24 ±0.03	0.66 ±0.03	3.2 ±0.4
6c	5-Et-5-DMTX	6.02 ±0.8	0.25 ±0.05	0.27 ±0.02	1.6 ±0.3
6d	5-Pr-5-DMTX	11.7 ±0.3	1.1 ±0.2	4.2 ±0.6	0.50 ±0.1
6e	5-Bu-5-DMTX	78.5 ±9	1.2 ±0.2	1.5 ±0.1	0.75 ±0.2

[a] Syntheses of **5a**, **5b**, **6a**, and **6b** were reported earlier [12]. [b] Methods described in [10]. [c] Averages of three or more evaluations with deviations from the average shown. [d] AM = aminopterin, MTX = methotrexate, DAM = deazaaminopterin, *etc.*

same effect as aminopterin; both, however, are less inhibitory (from 4- to 7-fold) than their lower 5-alkyl homologs **5b** and **5c**. Also against L1210 in culture, N^{10}-methyl compounds **6d** and **6e** are more inhibitory (by about 2-fold) than methotrexate, but, as with the N^{10}-H compounds, they are less inhibitory (by about 4-fold) than their 5-alkyl homologs **6b** and **6c**.

In the tests for growth inhibition of S180 cells, both **5d** and **5e** are less inhibitory than aminopterin (from 4- to 5-fold) and are much less inhibitory than the methyl and ethyl homologs **5b** and **5c** (8-fold less than **5b** and 17-fold less than **5c**). The N^{10}-methyl compounds are somewhat more inhibitory than methotrexate toward growth of S180 cells (2-fold for **6d**, 5-fold for **6e**), but higher homologs **6d** and **6e** are consistently less effective than the lower 5-alkyl homologs.

Also in Table 3 are results showing that the higher alkyl homologs, both the N^{10}-H and the N^{10}-methyl types, undergo carrier-mediated influx [9] through the L1210 cell membrane more readily than their parent compound and their lower alkyl homologs. The influx advantage is most pronounced in the comparisons of N^{10}-methyl derivatives **6d** and **6e** with methotrexate where the factors are 6- to 8-fold. Comparisons of the influx of **5d** and **5e** with **6d** and **6e** show the two types to be nearly identical whereas aminopterin has an influx advantage of at least 3-fold over methotrexate. The advantage might help account for the greater L1210 cell growth inhibitory effect of **6d** and **6e** compared with methotrexate despite the reduced inhibition of DHFR by these compounds. Similarly, N^{10}-H compounds **5d** and **5e** have transport advantages of about 2-fold over aminopterin, and, despite the diminution in inhibitory effect on the DHFR, the compounds exert about the same degree of L1210 cell growth inhibition as aminopterin.

Correlations of Biological Results and Molecular Graphics Studies.

Our molecular modeling studies suggest that the binding of the 5-alkyl-5-deaza analogues to human DHFR in many respects parallels the behavior expected from analysis of the binding modes of numerous ligands previously observed crystallographically in complexes with DHFR derived from human [14-17] and other sources [18-20]. Nevertheless, each of the three primary compounds mod-

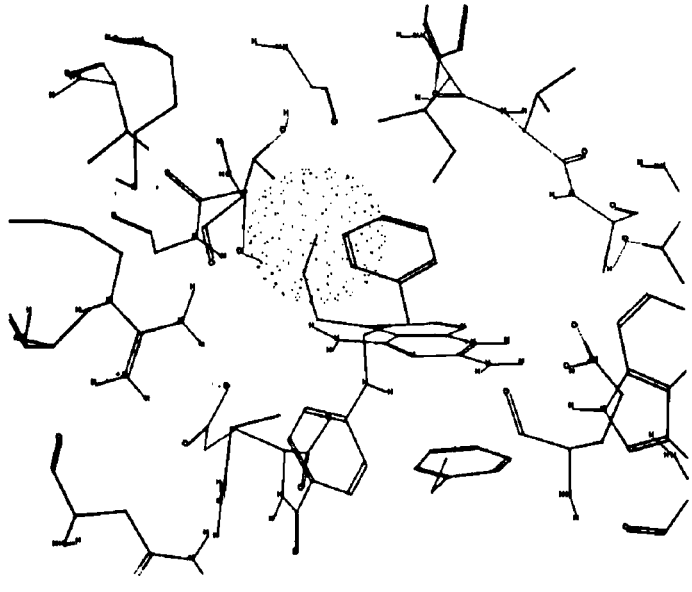


Figure 1

eled thus far (5-alkyl-5-DAM analogues **5b**, **5d**, and **5e**) displays unique ligand—enzyme interactions, resulting in a spectrum of binding modes differing in some details from our extrapolation of literature results.

Chief among the factors favorably affecting high affinity binding to DHFR are the hydrophobic interactions between the heteroaromatic and benzoyl rings of the inhibitor and the plenitude of nonpolar amino acids comprising much of the active site surface. The 5-propyl-5-deaza compound **5d** represents a typical case (Figure 1). Our model, not unexpectedly, reveals the potential for extensive van der Waals contact between the heterocyclic system and the two prominent aromatic active site residues, F31 and F34, which bracket the deazapteridine ring on either side. In contrast to our model of methotrexate, folate, and several other systems, the 5-propyl substituent begins to encroach upon F34 and disrupt its interaction with the ring, an effect which becomes more severe when the side chain is extended as in the butyl homolog **5e**. This encroachment in the 5-butyl compound has repercussions, which will be mentioned shortly, for other aspects of its binding to DHFR.

There are two other regions engaged in hydrophobic attraction in this complex. The benzoyl system is bounded by the aliphatic side chains of L22 and I60, and P61; in addition, there is some contact with the peptide backbone connecting residues 59-61. The 5-propyl substituent itself occupies a pocket surrounded by I16, V50, V115, and the methyl group of T56, as well as F34. This region adjoins the β-pleated sheet forming the core of the enzyme.

Two prominent salt bridges between enzyme and inhibitor contribute to the specificity of substrate binding in our model, concordant with expectations gleaned from analogous crystallographic studies [16-19]. Thus, the γ-carboxyl of the bound glutamate strongly hydrogen bonds to R70, while E30 abuts *N*-1 and the 2-amino moiety of the heterocyclic system. These latter groups also participate in an extensive hydrogen bonding scheme involving, in addition to E30, W24, T136, H_2O 401, and the deazapteridine *N*-8. The only other notable polar forces result from a hydrogen bond between the benzoyl carbonyl oxygen of the ligand, and the side chain amide proton of N64, and from the side chain of Q35 to the α-glutamyl carboxyl of the ligand. It is noteworthy that, in all complexes we have modeled except the 5-propyl species, it is the α-carboxyl which interacts with R70, not the γ-carboxyl, which in many analogs would be polyglutamylated intracellularly.

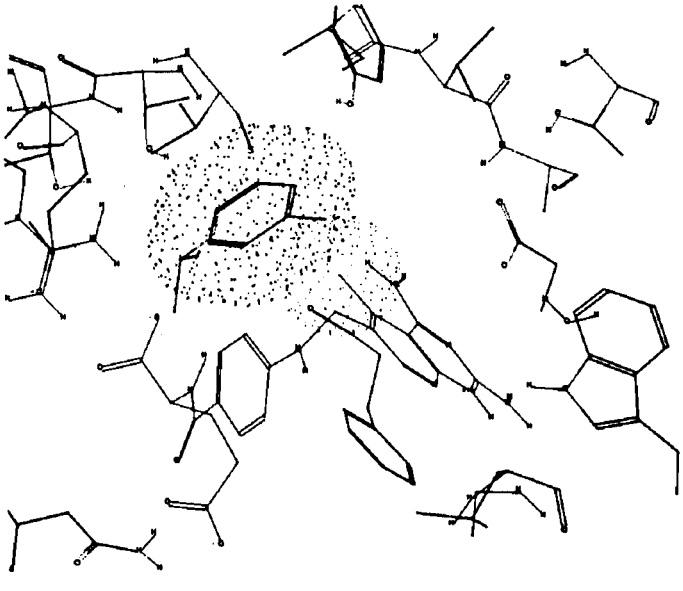


Figure 2

The slight reduction in binding affinity observed for the propyl and butyl derivatives may result in part from the altered interactions of the α- and γ-carboxylates with the enzyme. Thus, the rearrangement of F34 forced by introduction of a long 5-alkyl side chain appears to expand the binding pocket, both compromising the hydrophobic stacking interaction just described, and permitting formation of a new, *intra*molecular hydrogen bond between the *N*-4 amino proton and the glutamate carboxylate oxygen. This repositioning of the carboxylate reduces its interaction with R70.

Binding of the 5-methyl homolog **5b** is driven by the large van der Waals attraction between the methyl group and F34 (Figure 2); in this case, direct interaction between the aromatic ring of F34 and the heterocycle is completely lacking, although base stacking with F31 is still observed. While most interactions of the benzoyl and glutamyl fragments are unaffected, significant alterations occur in the binding mode of the pyrimidine substructure. Positioning of the heterocyclic methyl toward F34 results in elimination of the salt bridge between E30 and the *N*-1/*N*-2 system. The latter interaction is replaced by hydrogen bonds between E30 and the *N*-4 amino group (and also T136), while water 401 remains H-bonded to W24, E30, and the 2-amino function.

The model presented here highlights the variety of binding modes available to potential DHFR inhibitors, and shows how subtle structural changes combined with currently understood selectivity requirements allow the effective design of antifolate candidates at the level of the target enzyme DHFR.

EXPERIMENTAL

Construction of the Model DHFR•Inhibitor Complex.

Detailed information concerning construction of the human DHFR model, and its use in the analysis of binding interactions with various folate ligands, has been fully described elsewhere

[13]. In contrast to the previously modeled pteridine ring systems, however, minimization of the 5-deaza compounds using these procedures resulted in significant non-planarity of the B-ring, irrespective of the nature of the substituent at position 5. Variation of minimization conditions did not solve this problem, which we attribute to poor parameterization of this ring system in the MACROMODEL/AMBER force field. Consequently, torsional restraints were imposed on all heterocyclic ring bonds, forcing them to near planar values typical for this ring system as observed experimentally in X-ray crystallographic studies [14].

Since the incorporation of a 5-alkyl chain introduces additional torsional degrees of freedom compared with the previously modeled structures, the Monte Carlo search algorithm was modified accordingly, with up to 14 randomly selected degrees of freedom varies at each MC step. Furthermore, the model was also refined to include active site amino acid side chain conformations in the search, with as many as 16 sidechain torsions varied at each step. Other procedures followed the published methods.

Synthetic Procedures.

Examinations by thin-layer chromatography (tlc) were performed on Analtech precoated (250 μm) silica gel G(F) plates. High-performance liquid chromatography (hplc) assays were made with a Waters Associates ALC-242 liquid chromatograph equipped with an ultraviolet detector (254 nm) and a M-6000 pump using a 30 x 0.29 cm C_{18} μBondapak column. Purity assays, done by reversed-phase in the isocratic mode with a mobile phase consisting of acetonitrile (10 or 15% by volume) in 0.1 *M* sodium acetate (*p*H 3.6), showed target compounds **5c-e** and **6c-e** to be of 98% purity or higher. Unless other conditions are specified, evaporations were performed with a rotary evaporator and a water aspirator. Products were dried *in vacuo* (<1 mm) at 22-25° over phosphorus pentoxide and sodium hydroxide pellets. Final products were dried and then allowed to equilibrate with ambient conditions of the laboratory. Melting points were observed on a Mel-Temp apparatus. Spectral determinations and elemental analyses were performed in the Spectroscopic and Analytical Laboratories Section of Southern Research Institute under the direction of Dr. W. C. Coburn, Jr. The proton nuclear magnetic resonance (^{1}H nmr) spectra were determined with a Nicolet NMC 300 NB spectrometer using tetramethylsilane (TMS) as internal reference. Chemical shifts (δ) listed for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assigned structures. Mass spectra were recorded on a Varian MAT 311A mass spectrometer in the fast-atom-bombardment mode.

2-Amino-4-alkyl-6-chloro-3,5-pyridinedicarbonitriles **1c-e**.

These intermediates were prepared by an adaptation of the procedure of Schmidt and Junek [21] as reported earlier for homologs **1a** and **1b** [12]. The required trimethyl or triethyl ortho esters were obtained from commercial suppliers. Results are listed in Table 1.

2-Amino-4-alkyl-3,5-pyridinedicarbonitriles **2c-e**.

The procedure that follows is an adaptation of that reported for the preparation of **1a** [22]. It is an improvement over our earlier procedure used to prepare **1a** and **1b** [12] in which 5% palladium on barium carbonate was used as both catalyst and acid acceptor. The preparation for **2d** is illustrative of the improved procedure. A solution of **1d** (42.3 g, 0.193 mole) in *N,N*-dimethylformamide (600 ml) and triethylamine (70 ml) containing palladium chloride (1.1 g) was shaken on a Parr apparatus under hydrogen initially at 45 psi. In this run, an examination by tlc (cyclohexane-ethyl acetate, 1:1) after 16 hours revealed a dominant spot (R_f 0.54) due to **2d** along with a spot (R_f 0.74) due to unchanged **1d**. The mixture was filtered, fresh palladium chloride (1.1 g) was added, and treatment with hydrogen at 45 psi was resumed. After 3 hours, tlc showed absence of starting **1d**. The mixture was filtered, and the filtrate was concentrated (<1 mm, bath to 30°) to about 75-100 ml. Dilution with cold water (1 l) caused precipitation of **2d** (32.6 g, 91% yield), homogeneous by tlc. Additional data and results on **2c** and **2e** are given in Table 1.

2,4-Diamino-5-alkylpyrido[2,3-*d*]pyrimidine-6-carbonitriles **3c-e**.

The preparation of **3d** typifies the procedure. Anhydrous guanidine hydrochloride (6.15 g, 0.064 mole) and sodium methoxide (3.49 g, 0.065 mole) were combined in dry 2-(2-methoxyethoxy)ethanol (270 ml), and the mixture was stirred for about 0.5 hour before it was combined with a solution of **2d** (12.0 g, 0.064 mole) in 2-(2-methoxyethoxy)ethanol (335 ml). The stirred mixture was heated under nitrogen at 150-160° for 7 hours. This mixture was allowed to cool to about 110° while another solution of guanidine (one-half the previous amount) in 2-(2-methoxyethoxy)ethanol was prepared. The second guanidine solution was added, and heating at 150-160° was resumed. After 5 hours the mixture was allowed to cool, then concentrated (<1 mm) to a viscous mixture. Addition of cold water (500 ml) gave a solid. The dried solid (11.4 g) was dissolved in *N,N*-dimethylformamide, and the solution was swirled with silica gel (about 40 g of 60-200 mesh). Evaporation (<1 mm) gave a solid dispersion of crude product in silica gel. The dispersion was pulverized, dried further *in vacuo*, then applied to a column (9 x 50-cm) of silica gel (60-200 mesh) poured from chloroform. Gravity elution by chloroform-methanol (5:1) was performed. Homogeneous fractions (of tlc R_f 0.55 using chloroform-methanol, 5:1) were combined and evaporated to give pure **3d** (3.9 g). Results are listed in Table 1.

N-[4-[[(2,4-Diamino-5-alkylpyrido[2,3-*d*]pyrimidin-6-yl)methyl]amino]benzoyl]-L-glutamatic Acid Diethyl Esters **4c-e**.

The procedure for the preparation of propyl compound **4d** is typical. A mixture of **3d** (1.21 g, 5.30 mmoles) and diethyl *N*-(4-aminobenzoyl)-L-glutamate (2.33 g, 7.23 mmoles) in glacial acetic acid (250 ml) containing damp Raney nickel (about 8 g) was stirred under hydrogen (over water in a gas burette) at atmospheric pressure for approximately 4 hours until hydrogen absorption had ceased. The resulting solution was filtered from catalyst, and the filtrate was evaporated (bath 30°). The residue was dissolved in the minimum of ethanol (12-15 ml), and the stirred solution was gradually treated with 3% sodium carbonate solution to *p*H 7.8. The resulting solid was collected with the aid of cold water, dried, and dispersed onto silica gel (60-200 mesh) as described above for precursor **3d**. The dispersion was applied to a silica gel column (5 x 50-cm) poured from chloroform. Elution by gravity flow with chloroform-methanol (95:5) followed. After tlc showed that diethyl *N*-(4-aminobenzoyl)-L-glutamate and minor contaminants more mobile than product **4d** had been eluted, the system was switched to 85:15 chloroform-methanol. Homogeneous fractions (tlc R_f 0.5 using chloroform-methanol, 3:1) were combined and evaporated to give pure **4d**

(470 mg). Additional data are given in Table 1.

N-[4-[[(2,4-Diamino-5-alkylpyrido[2,3-*d*]pyrimidin-6-yl)methyl]amino]benzoyl]-L-glutamic Acids **5c-e**.

The following procedure for the conversion of **4c** to **5c** is typical. A suspension of **4c** (1.50 g, 2.87 mmoles) in methanol (270 ml) containing 1 *N* sodium hydroxide (7.2 ml) was stirred at 20-25° for 4 days. Solution occurred during this time. Progress of the hydrolysis was monitored by hplc as described earlier [12]. The solution was evaporated (bath 20-25°), and the residue was dissolved in water (40 ml). The clear solution (of *p*H 12) was stirred while being treated with 1 *N* hydrochloric acid to lower the *p*H to 3.7. Solid **5c** precipitated and was collected, washed with water, and dried (yield 1.20 g). Additional data are listed in Tables 1 and 2.

N-[4-[[(2,4-Diamino-5-alkylpyrido[2,3-*d*]pyrimidin-6-yl)methyl]methylamino]benzoyl]-L-glutamic Acids **6c-e**.

Reductive methylation of **5c-e** using formaldehyde and sodium cyanoborohydride was carried out using the same procedure and on a similar scale with that described in detail for the preparation of **6b** from **5b** [12]. Results from preparations of **6c-e** by this method are listed in Tables 1 and 2. The 5-ethyl compound **6c** was also prepared *via* its diethyl ester **10** as described below.

6-(Bromomethyl)-2,4-diamino-5-ethylpyrido[2,3-*d*]pyrimidine (**9**).

This compound was prepared from **3c** in three steps as indicated in Scheme 1 using the procedures reported earlier for preparing the corresponding 5-methyl compound [24]. The nitrile **3c** (7.2 g) was converted to aldehyde **7** (4.0 g, 55% yield; ms: mz 218, MH$^+$ for $C_{10}H_{11}N_5O$) which was reduced to hydroxymethyl compound **8** (69% yield; ms: mz 220, MH$^+$ for $C_{10}H_{13}N_5O$). This sample (2.8 g) was treated with dry hydrogen bromide in acetic acid as described for its 5-methyl homolog to give 5.3 g of 9 hydrobromide solvated by acetic acid (93% yield based on formulation shown below); ms: m/z 282 and 284, MH$^+$ for $C_{10}H_{12}BrN_5$; ^{1}H nmr: δ 1.24 (t, CH_3CH_2), 3.24 (q, CH_2CH_3), 4.94 (s, CH_2Br), 8.80 (s, C^7-H); presence of acetic acid evidenced by singlet at δ 1.90. The molar ratio of **9** to acetic acid is 1:0.15.

Anal. Calcd. for $C_{10}H_{12}BrN_5 \cdot 0.15CH_3CO_2H \cdot 1.7HBr$: C, 28.85; H, 3.36; N, 16.33. Found: C, 28.70; H, 3.63; N, 16.36.

Diethyl *N*-[4-[[(2,4-Diamino-5-ethylpyrido[2,3-*d*]pyrimidin-6-pyrimidinyl)methyl]methylamino]benzoyl]-L-glutamate (**10**).

Diethyl *N*-[4-(methylamino)benzoyl]-L-glutamate [12,23] (673 mg, 2.0 mmoles) and **9** (808 mg, 1.87 mmoles based on formula given under preparation above) were dissolved in *N,N*-dimethylacetamide (20 ml). The solution was immediately treated with calcium carbonate (270 mg, 2.70 mmoles). The resulting mixture was stirred at 20-25° under nitrogen in a stoppered flask wrapped in aluminum foil. After 7 days, the mixture was filtered, and the solvent was removed (<1 mm, bath to 30°). The gummy residue was dissolved in methanol (75 ml) for dispersion onto silica gel (3.0 g of 60-200 mesh). Following evaporation of methanol, the dispersion was applied atop a column of silica gel (approximately 300 ml of 230-400 mesh) poured from chloroform-methanol (7:1). Gravity elution with the same solvent gave fractions homogeneous in product having tlc R_f 0.4 (chloroform-methanol, 5:1). The combined fractions were evaporated to give **10**, yield 450 mg (42% yield); ms: m/z 538, MH$^+$.

Anal. Calcd. for $C_{27}H_{35}N_7O_5 \cdot 2.5H_2O$: C, 55.66; H, 6.92; N, 16.83. Found: C, 55.66; H, 6.70; N, 17.19.

N-[4-[[(2,4-Diamino-5-ethylpyrido[2,3-*d*]pyrimidin-6-yl)methyl]methylamino]benzoyl]-L-glutamic Acid (**6c**) from **10**.

Hydrolysis of **10** as described above for the conversion of **4c** to **5c** gave pure **6c**•2H_2O in 71% yield (321 mg from 425 mg, 0.730 mmole, of **10**•2.5H_2O); ms: m/z 482, MH$^+$; ^{1}H nmr and hplc results same as those of **6c** prepared from **5c** (see Table 2).

Anal. Calcd. for $C_{23}H_{27}N_7O_5 \cdot 2H_2O$: C, 53.38; H, 6.04; N, 18.94. Found: C, 53.57; H, 5.97; N, 19.10.

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