Effect of C9-Methyl Substitution and C8-C9 Conformational Restriction on Antifolate and Antitumor Activity of Classical 5-Substituted 2,4-Diaminofuro[2,3-*d*]pyrimidines[1]

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N-[4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**5**) and its C8-C9 conformationally restricted *E*- and *Z*-isomers (**6** and **7**) were designed and synthesized in order to investigate the effect of incorporating a methyl group at the C9 position and of conformational restriction at the C8-C9 bridge of *N*-[4-[2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**1**) with respect to dihydrofolate reductase (DHFR) inhibitory activity as well as antitumor activity. The compounds were synthesized by a Wittig reaction of 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine with ethyl 4-acetylbenzoate followed by catalytic reduction, hydrolysis, and standard peptide coupling with diethyl L-glutamate. The biological results indicated that the addition of a 9-methyl group to the C8-C9 bridge, as in **5**, increased recombinant human (rh) DHFR inhibitory potency (IC_{50} = 0.42 μM) as well as the potency against the growth inhibition of tumor cells in culture (CCRF-CEM EC_{50} = 29 nM, A253 EC_{50} = 28.5 nM, and FaDu EC_{50} = 17.5 nM) compared with the 9-desmethyl analogue **1.** However, the conformationally restricted 4:1 *Z*/*E* mixture of **7** and **6** was less potent than **5** in both assays, and the pure *E*-isomer **6** was essentially inactive. These three classical analogues were also evaluated as inhibitors of *Lactobacillus casei*, *Escherichia coli*, and rat and rh thymidylate synthase (TS) and were found to be weak inhibitors. All three analogues **5**−**7** were good substrates for human folylpolyglutamate synthetase (FPGS). These data suggested that FPGS is relatively tolerant to different conformations in the bridge region. Further evaluation of the cytotoxicity of **5** and **7** in methotrexate (MTX)-resistant CCRF-CEM cell sublines suggested that polyglutamylation was crucial for their mechanism of action. Metabolite protection studies of **5** implicated DHFR as the primary intracelluar target. Compound **5** showed GI_{50} values in 10^{-9}−10^{-7} M range against more than 30 tumor cell lines in culture.

Introduction

Folate metabolism has been an attractive chemotherapeutic target due to its crucial role in the biosynthesis of nucleic acid precursors.[2] Tetrahydrofolate (FH_4), the key component of folate metabolisms, serves as a cofactor precursor to carry one-carbon units for the biosynthesis of purines and deoxythymidylate (dTMP). During the synthesis of dTMP, FH_4 is oxidized to 7,8-dihydrofolate (FH_2) and must be converted back to the reduced form by dihydrofolate reductase (DHFR) to continue DNA synthesis.[3] Inhibition of DHFR depletes the FH_4 pool; thus DHFR inhibitors have found clinical utility as antitumor, antibacterial, and antiprotozoan agents.[4,5]

As part of a continuing effort to develop novel classical antifolates as DHFR inhibitors and as antitumor agents, Gangjee et al.[6,7] previously reported the synthesis of *N*-[4-[2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**1**) and its $-CH_2NH-$, $-CH_2N(CH_3)-$, and $-CH_2NHCH_2-$ analogues **2**−**4**. The three-atom bridged compound **4** was poorly active against DHFR (IC_{50} = 1.0 × 10^{-5} M), whereas the three two-atom bridged analogues **1**−**3** showed moderate to high potency (IC_{50} = 1.0 × 10^{-8} to 1.0 ×10^{-6} M). These analogues were also potent inhibitors of tumor cell growth in culture (EC_{50} = 1.0 ×10^{-8} to 1.0 × 10^{-7} M). The C9 analogue **1** (EC_{50} = 1.0 × 10^{-7} M) was about one-half as potent as its 9-aza analogue **2** against cultured CCRF-CEM cells, whereas the N9-methyl analogue **3** was about twice as potent.

Molecular modeling using Sybyl 6.2[8] and the crystal structure of recombinant human DHFR (rhDHFR)[9] with methotrexate (MTX) in place of folate indicates that the N9-methyl moiety of **3** (superimposed on MTX) is positioned to interact with the hydrophobic Val115 residue of the enzyme active site. Thus substitution of a methyl group at C9 of **1** would be predicted to enhance binding and hence the inhibition of DHFR. In addition, molecular modeling indicated that C9-methyl group could introduce conformational restriction about the C8-C9 bond thus decreasing the number of accessible low-energy conformations compared with **1**. Similar substitutions of a methyl group in the C9-C10 bridge region of 6-6 fused analogues have been reported by DeGraw et al.[10,11a,b] in 2,4-diaminopteridine and 2,4-diaminopy-

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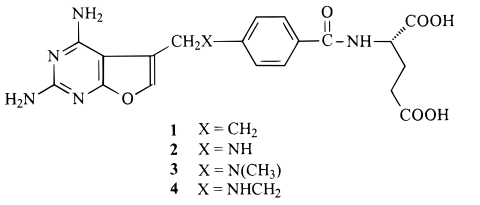

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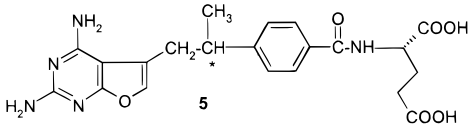
4 X = $NHCH_2$



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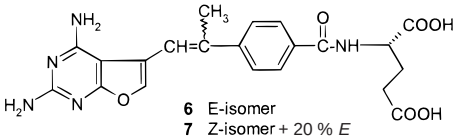


6 E-isomer
7 Z-isomer + 20 % *E*

rido[2,3-*d*]pyrimidine analogues which enhanced inhibitory activity against tumor cells in culture. As part of a structure−activity relationship (SAR) study on the relative conformational orientation of the furo[2,3-*d*]-pyrimidine ring with respect to the benzoylglutamate side chain of the two-atom bridged classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine antifolates, we designed and synthesized *N*-[4-[1-methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**5**), the 9-methyl analogue of **1**.

A number of classical antifolate C9-C10 bridged analogues have been reported in the 6-6 fused bicyclic sytems, such as pteridines,[10] pyrido[2,3-*d*]pyrimidines,[11,12] and pyrido[3,2-*d*]pyrimidines,[13] and C8-C9 bridged analogues in the 6-5 fused bicyclic systems, such as pyrrolo-[2,3-*d*]pyrimidines,[14] cyclopenta[*d*]pyrimidines,[15] and furo[2,3-*d*]pyrimidines.[6] There is however a dearth of information regarding the *E*- and *Z*-isomers of these C-C bridged classical analogues. The sole report of characterized geometric isomers is by Harris et al.[16] who reported that in nonclassical 6-substituted 2,4-diaminoquinazolines, the *Z*-isomer was more favorable for bacterial DHFR inhibition than the *E*-isomer and was similar in activity to the reduced bridge analogue. It is surprising to note that despite the synthetic methodology which affords the unsaturated precursors, none of the geometrically restricted, unsaturated, classical analogues, to our knowledge, were evaluated for DHFR inhibitory activity or for their inhibition of the growth of tumor cells in culture. Thus it was of considerable of interest to synthesize and isolate the *E*/*Z* mixture and separate the *E*- and *Z*-isomers of **5** for biological evaluation against DHFR and against the growth of tumor cells in culture. Thus the C9-methylated C8-C9 conformationally restricted *E*-**6** and *Z*-**7** *N*-[4-[1-methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethenyl]benzoyl]-L-glutamic acids were designed as classical antifolates and as antitumor agents.

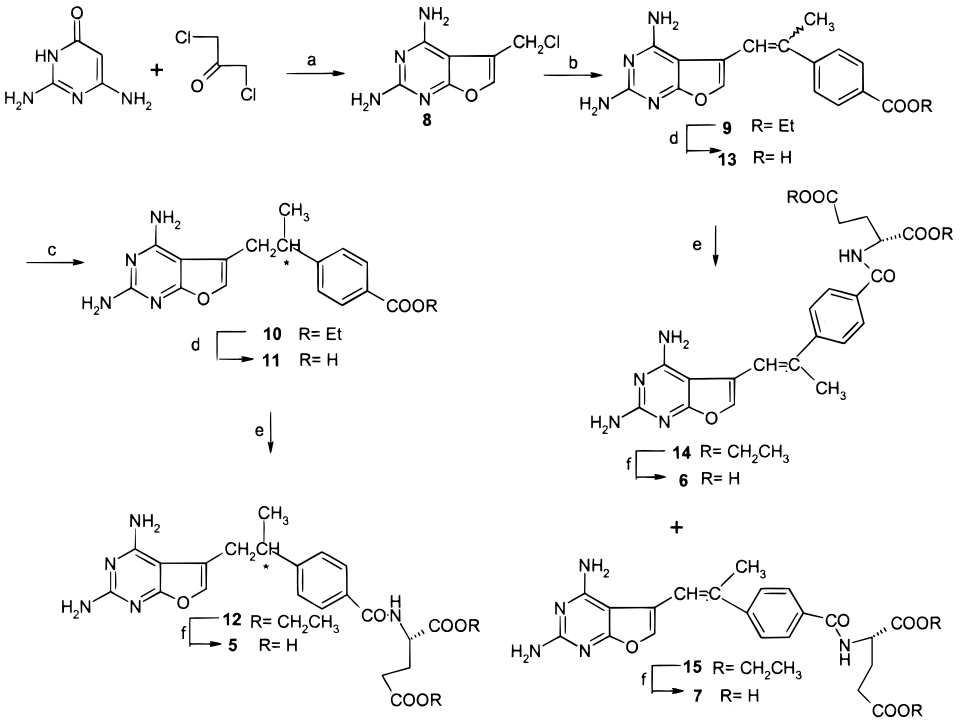
Polyglutamylation via folylpolyglutamate synthetase (FPGS) is an important mechanism for trapping classical folates and antifolates within the cell thus maintaining high intracellular concentrations and in some instances for increasing binding affinity for folate-dependent enzyme.[17a,b] It was of interest to determine the effect of adding a C9-methyl and C8-C9 bond restriction with respect to FPGS activity as well. In addition, thymidylate synthases (TS), a crucial enzyme in the biosynthesis of DNA which catalyzes the sole de novo synthesis of dTMP from dUMP, is a folate-cofactor-requiring enzyme.[18] It was also of interest to evaluate these new classical furo[2,3-*d*]pyrimidines **5**−**7** as inhibitors of TS.

Chemistry

The synthetic route to the target molecules **5**−**7** is outlined in Scheme 1. A modification of the methodology reported previously[6] was utilized to assemble the skeleton of the two-carbon bridged target **5**. The crucial intermediate **8** was readily obtained by the reaction of 2,6-diaminopyrimidin-4-one and dichloroacetone in DMF at room temperature as previously reported.[19] Wittig condensation of **8** and ethyl 4-acetylbenzoate in DMSO solution, using excess *n*-Bu_3P (3 equiv) and NaH (2.2 equiv) to form the ylide and methanol to quench the reaction, furnished the olefin **9** in 42% yield as a mixture of *E*- and *Z*-isomers. The mixture of the *E*- and *Z*-isomers was established from its ^{1}H NMR where the characteristic C6 proton signal was observed at 7.52 ppm for the *E*-isomer, which is 0.85 ppm downfield from the C6 proton of the *Z*-isomer (6.67 ppm). This compares favorably with previously reported *E*- and *Z*-products.[6] During the synthesis, some methyl ester exchange with ethyl ester was detected in the ^{1}H NMR spectrum. In addition the removal of excess tributylphosphine and phosphine oxide was also problematic. In an attempt to improve the yield of **9**, the amounts of tributylphosphine and NaH were reduced to 1.5 equiv each and ethanol was used in the workup instead of methanol. This afforded the olefin in 65% yield as a mixture (*E*:*Z* ratio 2:1) as determined by ^{1}H NMR. Catalytic hydrogenation of **9** under optimized conditions followed by column chromatography and recrystallization from MeOH afforded the 8,9-dihydro derivative **10** in 75% yield. Saponification of **10** with aqueous sodium hydroxide afforded the free acid **11** in 90% yield. Subsequent coupling of **11** with diethyl L-glutamate using isobutyl chloroformate as the coupling agent afforded the product **12** in 85% yield. Final saponification with aqueous sodium hydroxide at room temperature followed by acidification to pH 4 in an ice bath afforded the diacid **5** in 97% yield. The structures of **5** and all intermediates were confirmed by ^{1}H NMR and elemental analysis.

It was more efficient to separate the *E*-isomer from the mixture after coupling with the L-glutamate diester. Thus hydrolysis of **9** with aqueous sodium hydroxide afforded the free acid **13** of the *E*:*Z* mixture in 92% yield. Subsequent coupling of **13** with diethyl L-glutamate using isobutyl chloroformate afforded the desired coupled products **14** and **15** as an *E*:*Z* mixture in 85% yield. All attempts to separate the *E*-isomer **14** from the *Z*-isomer **15** using column chromatography were unsuccessful. Partial separation was finally achieved by fractional recrystallization from methanol/ethyl acetate (1:1). The white crystals which separated contained the pure *E*-isomer as confirmed by TLC and by its characteristic C6 proton at 7.50 ppm. To further characterize the structure of the *E*-isomer, a 500-MHz two-dimensional proton−proton ROESY experiment was performed, and the spectrum clearly indicated that the C6 proton correlated to the C9-methyl group while the C8 proton

Scheme 1[a]



[a] Reagents: (a) DMF, rt; (b) (i) Bu_3P/DMSO, (ii) NaH, (iii) ethyl 4-acetylbenzoate; (c) 5% Pd−C/H_2, 45 psi, MeOH/$CHCl_3$/DMF; (d) (i) 1 N NaOH, DMSO/MeOH, (ii) 1 N HCl; (e) iBuOCOCl, Et_3N, diethyl L-glutamate; (f) (i) 1 N NaOH, DMSO/MeOH, (ii) 1 N HCl.

Table 1. Inhibitory Concentration (IC_{50}, μM) against Isolated DHFR and TS

	DHFR				TS			
compd	rh	lc	ec	tg	rh	lc	ec	rat
5	0.42	2.1	1.1	2.1	>360	>360	100	>360
6	42	84	11	110	>360	>360	>360	>360
7[a]	2.2	4.4	1.1	4.4	>360	>360	>360	>360
1[b]	1.0	0.1	nd	nd	220	200	nd	nd
2[c]	0.45	0.56	nd	0.70	>200	>200	nd	nd
3[c]	0.22	0.22	nd	19.8	>200	>200	nd	nd
MTX	0.022	0.011	0.009	0.022				
PDDF					0.15	0.055	0.1	0.36

[a] Compound **7** is a 1:4 *E:Z* mixture; [b] Data derived from ref 6. [c] Data derived from ref 7.

correlated with the 4-NH_2 and the side chain phenyl proton. This uneqivocally demonstrated the *E*-geometry for compound **14**. The *Z*-isomer remained in the filtrate along with residual *E*-isomer. Separation of the pure *Z*-isomer from this mixture was unsuccessful and it was decided to carry this mixture to the next step. Final saponification of pure **14** with aqueous sodium hydroxide at room temperature followed by acidification to pH 4 in an ice bath afforded the *E*-diacid **6** in 97% yield. The *Z*-diacid **7** obtained in 95% yield after saponification of **15** was contaminated with about 20% of **6** as indicated by the ^{1}H NMR on the basis of the C6 proton (*Z* at 6.64 ppm and *E* at 7.50 ppm) integrations of the mixture.

It should be noted that in our synthetic sequence, the catalytic reduction of **9** using palladium on activated carbon afforded **10** as a *R,S* racemic mixture at C9 which generated **5** as a mixture of diastereomers at C9. Attempts to separate the diastereomers using column chromatography were unsuccessful.

Biological Evaluation and Discussion

The classical analogues **5** and **6** and the 80/20% *Z/E* mixture **7** were evaluated as inhibitors of *Lactobacillus casei* (lc), *Escherichia coli* (ec), *Toxoplasma gondii* (tg), and recombinant human (rh) DHFR.[20−22] The inhibitory potency (IC_{50}) values are listed in Table 1 and compared with the value for methotrexate (MTX) and the previously reported values for **1**−**3.**[6,7] The reduced analogue **5** inhibited rhDHFR with an IC_{50} = 0.42 μM and was twice as potent as its 9-CH_2 analogue **1**. This inhibitory activity of **5** is about one-twentieth that of MTX and is similar to that of the 9-NH analogue **2** and about one-half that of the N9-CH_3 analogue **3**. The 80/20% *Z/E* mixture **7** was 5-fold less potent than the reduced analogue **5**, while the *E*-isomer was only marginally active against rhDHFR with an IC_{50} of 42 μM. In contrast to analogue **1**, which had a 10-fold increased potency against lcDHFR compared with rhDHFR, all three analogues **5**−**7** were more potent against rhDHFR than lcDHFR or tgDHFR. Compound **5** was also more potent against rhDHFR than ecDHFR. Thus compared to the 9-desmethyl analogue **1**, introduction of a 9-CH_3 moiety not only increases potency but also reverses selectivity for rhDHFR compared to lcDHFR. The bacterial DHFR inhibitory activity of these classical analogues **5**−**7** (Table 1) parallels that reported by Harris

Table 2. Activity of **5**−**7** as Substrates for rhFPGS[a]

substrate	K_m, μM	V_{max}, rel	V_{max}/K_m	*n*
aminopterin	4.5 ± 0.9	1	0.23 ± 0.05	6
5	1.2 ± 0.3	0.52 ± 0.03	0.46 ± 0.09	2
6	5.3 ± 0.8	0.76 ± 0.01	0.15 ± 0.02	2
7[b]	10.3 ± 0.2	0.56 ± 0	0.05 ± 0	2
1[c]	8.5 ± 2.1	0.65 ± 0.01	0.07 ± 0.02	3

[a] FPGS substrate activity was determined as described in the Experimental Section. Values presented are average ± SD if $n \geq 3$ and average ± range for $n = 2$. V_{max} are calculated relative to aminopterin within the same experiment. [b] Compound **7** is a 1:4 *E:Z* mixture. [c] Data from ref 6.

et al.[16] for nonclassical 2,4-diamino-6-substituted quinazolines in that the C8-C9 reduced analogue is equipotent with the *Z*-isomer and the *E*-isomer has significantly reduced activity.

Analogues **5**−**7** were also evaluated as inhibitors of lcTS, ecTS, rat (r) TS, and rhTS [23,24] and were inactive or marginal inhibitors of these enzymes with IC_{50} values > 360 μM in most cases (Table 1) compared to N10-propargyl-5,8-dideazafolate (PDPF) as a control.

Since polyglutamylation plays a role in the mechanism of action of some classical antifolates,[17a,b] it was of interest to evaluate these analogues as substrates for human FPGS (Table 2). The 9-methyl analogue **5** was about twice as efficient (higher V_{max}/K_m) as aminopterin (AMT) as an FPGS substrate; **5** also had a 7-fold lower K_m and 7-fold higher substrate efficiency relative to its unmethylated parent **1**.[6] Since **5** is a mixture of diastereomers at C9, it will be necessary to determine the activity of each diastereomer to determine the effect of chirality at C9 on FPGS activity. Of interest is the finding that C9 methylation (**1** vs **5**) is highly beneficial with regard to FPGS activity. This is in contrast to a related series of C8-N9-containing analogues, N9 methylation is slightly deterimental (**2** vs **3**;[7]) to FPGS activity. The C8-C9 conformationally restricted *E*-isomer **6** possessed substrate efficiency similar to that of AMT but was 3-fold less efficient than that of the unrestricted **5**. Interestingly **6** was still more efficient than unmethylated, unrestricted **1** (K_m, 8.5; V_{max}/K_m, 0.07[6]). The conformationally restricted *Z:E* (4:1) mixture **7** was a less efficient FPGS substrate than was the pure *E*-isomer **6**, primarily through an increase in K_m; however **7** was still about as efficient as unmethylated, unrestricted **1**. These data suggest that FPGS is relatively tolerant to different conformations in the C8-C9 bridge region. These data further suggest polyglutamylation may be important in the mechanism of action of these novel furo[2,3-*d*]pyrimidines.

The target compounds **5**−**7** were also evaluated as inhibitors of the growth of CCRF-CEM human leukemia cells in culture during continuous exposure (Table 3).[25−27] The reduced compound **5** was highly cytotoxic against CCRF-CEM cells, had a potency similar to MTX, and was about 10-fold more potent than **1**, 5-fold more potent than **2**, and equipotent with **3**. This indicates that 9-methylation (as in **3** and **5**) is highly conducive to tumor growth inhibition in culture. The pure conformationally restricted *E*-isomer **6** was a very weak inhibitor of CCRF-CEM cells growth, while analogue **7**, which is 80% *Z*-isomer, was moderately inhibitory. These data suggest the *Z*-isomer is the only one in the *E*/*Z* pair which is biologically active. These three classical analogues were further evaluated as inhibitors of the growth of A253 and FaDu squamous cell carcinoma of the head and neck cell lines in culture, during continuous exposure (Table 3). The inhibitory activities of **5**−**7** were consistent with the results obtained against the CCRF-CEM cells. The most potent compound was the reduced analogue **5** which was similar to MTX; the 80% *Z*-isomer **7** was much less potent and the *E*-isomer **6** was inactive. The activity of **5** further underscores the importance of a 9-CH_3 reduced bridge to tumor cell inhibition in culture.

Since compound **5** was 19-fold less potent than MTX against isolated rhDHFR but only about 2-fold less potent against the growth of tumor cells (CCRF-CEM, FaDu, and A253) in culture, it was of interest to further define its mechanism of action. Compounds **5** and **7** were thus evaluated as inhibitors of the growth of three CCRF-CEM sublines with defined mechanisms of resistance to MTX.[25−29] Against the increased DHFR subline R1, compounds **5** and **7** were both about 20-fold less potent than they were against parental cells. Thus these compounds were qualitatively similar to MTX, though for the latter drug this difference was 48-fold. Despite their relatively low potency (compared to MTX) as inhibitors of purified rhDHFR (Table 1), these data are consistent with DHFR being the primary target of these analogues. The MTX transport-deficient subline R2 is cross-resistant to both **5** and **7** indicating that both use the MTX/reduced folate carrier (RFC) for uptake. However, the degree of cross-resistance to **5** (15-fold) and **7** (8-fold) was much less than to MTX (111-fold) which may suggest that the mutation in the MTX/RFC

Table 3. Growth Inhibition of CCRF-CEM Human Leukemia Cells, Its MTX-Resistant Sublines, Human Squamous Cell Carcinoma FaDu, and A253 Cells during Continuous Exposure (0−12 h)[a]

cell line	resistance mechanism	MTX	EC_{50}, nM					
			5	**6**	**7**[b]	**1**[c]	**2**[d]	**3**[d]
CCRF-CEM	sensitive	14.4 ± 1.0 (*n* = 5)	29.2 ± 3.3 (*n* = 5)	34000 ± 6000 (*n* = 2)	1175 ± 83 (*n* = 4)	290 ± 10 (*n* = 3)	142 ± 11 (*n* = 4)	47 ± 7 (*n* = 4)
R1	increase DHFR	675 ± 35 (*n* = 2)	685 ± 45 (*n* = 2)	nd[e]	32000 ± 1000 (*n* = 2)	nd[e]	1523 ± 196 (*n* = 3)	807 ± 68 (*n* = 3)
R2(Bos)	deficient in transport	1600 ± 100 (*n* = 2)	430 ± 60 (*n* = 2)	nd[e]	17000 ± 0 (*n* = 2)	nd[e]	nd[e]	nd[e]
R30dm	decrease FPGS	14 ± 0 (*n* = 2)	220 ± 20 (*n* = 2)	nd[e]	6400 ± 900 (*n* = 2)	425 ± 50 (*n* = 2)	663 ± 66 (*n* = 3)	1030 ± 510 (*n* = 3)
FaDu	sensitive	11.3 ± 1.8 (*n* = 2)	17.5 ± 0.5 (*n* = 2)	>10000 (*n* = 2)	1400 ± 100 (*n* = 2)	180 ± 20 (*n* = 2)	126 ± 34 (*n* = 2)	55 ± 15 (*n* = 2)
A253	sensitive	14.5 ± 0.5 (*n* = 2)	28.5 ± 2.5 (*n* = 2)	>10000 (*n* = 2)	700 ± 70 (*n* = 2)	540 ± 90 (*n* = 3)	nd[e]	nd[e]

[a] EC_{50} = concentration of drug required to decrease cell viability as measured by MTA (MTT assay) by 50% after 5 days of treatment. [b] Compound **7** is a 1:4 *E:Z* mixture. [c] Data from ref 6. [d] Data from ref 7. [e] nd = not determined.

Table 4. Protection of FaDu Cells from the Growth Inhibitory Effects of MTX and **5** by 50 μM Hx, 40 μM TdR, or the Combination of 50 μM Hx + 40 μM TdR[a]

	relative growth, % of control			
drug	no addn	Hx	TdR	Hx + TdR
MTX	5.75 ± 1.25	11.25 ± 5.75	9.25 ± 1.75	102 ± 3.0
5	5.75 ± 0.75	3.75 ± 0.75	8.0 ± 1.5	105.25 ± 4.75

[a] Protection was determined as described in the Experimental Section. Data are the average ± range of duplicate determinations within a single experiment. The entire experiment was repeated with similar results. Metabolites themselves had no effect on the cell growth.

affects transport of 2,4-diaminofuro[2,3-*d*]pyrimidines less than it does 2,4-diaminopteridines. Alternatively, a second route for transport of 2,4-diaminofuro[2,3-*d*]-pyrimidines may become available at higher concentrations. It is also possible, however, that **5** and **7** are as poorly transported as is MTX, but these analogues are more readily converted to longer ($Glu_{\geq 3}$), more potent polyglutamate inhibitors and that this more efficient metabolism compensates for the poor transport. Kinetic constants for monoglutamyl **5** and **7** with human FPGS (which primarily measures conversion to the diglutamate) were determined (Table 2); the efficiency of conversion to longer polyglutamates is under study. The cross-resistance of the FPGS-deficient subline R30 to **5** (8-fold) and **7** (5-fold) in continuous exposure suggests that polyglutamylation plays a role in the mechanism of action of these analogues, probably by increasing their affinity for their target enzyme, DHFR. These data are consistent with those (Table 2) showing that the analogues are substrates for FPGS. Although DHFR is often insensitive to the polyglutamylation status of substrates and inhibitors,[30] enhanced potency of polyglutamates of a few novel antifolates against DHFR has been reported.[31]

Metabolite protection studies were carried out with **5** in order to further elucidate its mechanism of action. Both **5** (at 100 or 200 nM) and MTX (at 40 and 50 nM), drug levels that inhibited growth by 92−98%, were fully protected by as little as 0.1 μM leucovorin indicating that **5** acts as an antifolate (data not shown). Addition of a combination of 40 μM thymidine (TdR) + 50 μM hypoxanthine (Hx) was required to fully protect against growth inhibition by **5** and by MTX (Table 4). With either TdR or Hx alone, only marginal protection was obtained. These results implicate DHFR as the primary intracelluar target of **5** and are consistent with the cross-resistance studies (above) and with previous data on the classical furo[2,3-*d*]pyrimidine antifolates **1**−**3**.[6,7]

Compound **5** was selected by the National Cancer Institute (NCI)[32] for evaluation in its in vitro preclinical antitumor screening program. The ability of compound **5** to inhibit the growth of 54 tumor cell lines was measured as GI_{50} values, the concentration required to inhibit the growth of tumor cells in culture by 50% as compared to a control. In more than 30 cell lines, compound **5** showed GI_{50} values in the 10^{-9}−10^{-7} M range (Table 2 in Supporting Information). It is noteworthy that in all leukemia as well as a variety of other cell lines, compound **5** had GI_{50} values in the nanomolar range or less. It was also interesting to note that **5** was not a general cell poison but showed selectivity both within a type of tumor cell line as well as across different tumor cell lines, with inhibitory values which in some instances differed by 10 000-fold. This compound is currently under further evaluation by the NCI as an antitumor agent.

In summary, three classical 5-substituted, 2,4-diaminofuro[2,3-*d*]pyrimidine antifolates (**5**−**7**) modified in the bridge region linking the furo[2,3-*d*]pyrimidine ring and the side chain benzoyl-L-glutamic acid were designed and synthesized, where the bridge flexibility is partially restricted with a C9-methyl substitution (compound **5**) and completely restricted by a double bond (compounds **6** and **7**). The biological results indicated that incorporating a C9-methyl group in the C8-C9 bridge region (analogue **5**) increases DHFR inhibitory activity and inhibition of the growth of CCRF-CEM leukemic as well as FaDu and A253 cells in culture compared to the C9 unsubstituted analogue **1,** as well as compared with the C8-N9 analogues **2** and **3**. Compared to **5**, the conformationally restricted 80/20% *Z*/*E* mixture **7** was much less potent and the pure *E*-isomer **6** was essentially inactive against rhDHFR and against the growth of CCRF-CEM cells in culture. This suggests that the conformation about the C8-C9 bridge of the classical 5-substiuted 2,4-diaminofuro[2,3-*d*]pyrimidines is an important determinant for DHFR and tumor inhibitory activity. The C8-C9 double bond which provides complete restriction is detrimental to DHFR inhibitory activity and to the inhibition of the growth of tumor cells in culture compared to the C8-C9 reduced analogue **5** where the C8-C9 bridge maintains some conformational flexibility.

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in an Abderhalden drying apparatus over P_2O_5 and refluxing ethanol. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, t = triplet, q = quartet, m = mutiplet, br = broad singlet. The relative intergrals of peak areas agreed with those expected for the assigned structures. Two-dimensional proton−proton ROESY experiment was performed on a Bruker DRX500 (500 MHz) spectrometer. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254- and 366-nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on 230−400 mesh silica gel purchased from Aldrich, Milwaukee, WI. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within ±0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates were not prevented despite 24−48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. and Fisher Scientific and were used as received.

Ethyl 4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethenyl]benzoate (9). To a solution of 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (**8**)[18] (1 g, 5 mmol) in anhydrous DMSO (15 mL) was added tributylphosphine (92%, 1.6 g, 7.5 mmol), and the resulting mixture was stirred at 60 °C in an oil bath for 3 h under N_2 to form the phosphonium salt. The deep orange solution was then cooled to room

temperature. To this solution was added sodium hydride (92% dispersion in mineral oil, 0.2 g, 7.5 mmol), followed by ethyl 4-acetylbenzoate (1.0 g, 5.5 mmol). The reaction mixture was stirred at room temperature for 38 h. TLC showed that the disappearance of the starting material (R_f 0.45) and appearance of two spots at 0.58 and 0.60 ($MeOH/CHCl_3$, 1:5, plus 1 drop of ammonia). The reaction was quenched with ethyl acetate (25 mL), chloroform (25 mL) and 50 mL ethanol, the resulting solution was evaporated and 100 mL methanol was added to dissolve the residue, followed by 10 g silica gel. This mixture was evaporated under reduced pressure to dryness to afford a silica gel plug which was loaded on a dry silica gel column (4 × 20 cm) and flash chromatographed initially with $CHCl_3$ (300 mL), then sequentially with 300 mL 2−5% MeOH in $CHCl_3$. Fractions which showed the major spot at R_f 0.60 along with the spot at R_f 0.58 were pooled and evaporated to dryness. Recrystallization from ethyl acetate afforded 1.35 g (65%) of **9** (*Z*:*E* ratio 1:2) as yellow needles: mp 222−226 °C; ^{1}H NMR (DMSO-d_6) *E*-isomer δ 1.28−1.35 (t, 3 H, OCH_2CH_3), 2.25 (s, 1 H, 9-CH_3), 4.28−4.35 (q, 2 H, OCH_2CH_3), 6.08 (s, 2 H, 4-NH_2), 6.53 (s, 2 H, 2-NH_2), 7.01 (s, 1 H, 8-CH), 7.52 (s,1 H, 6-CH), 7.78−7.81 (d, 2H, 3′-, 5′-CH), 7.94−7.97 (d, 2 H, 2′-, 6′-CH); *Z*-isomer δ 1. 28−1. 35 (t, 3 H, OCH_2CH_3), 2.20 (s, 3 H, 9-CH_3), 4. 28−4.35 (q, 2 H, OCH_2CH_3), 6.02 (s, 2 H, 4-NH_2), 6.32 (s, 1 H, 8-CH), 6.54 (s, 2 H, 2-NH_2), 6.67 (s, 1 H, 6-CH), 7.33−7.36 (d, 2 H, 3′-, 5′-CH), 7.88−7.91 (d, 2 H, 2′-, 6′-CH). Anal. ($C_{18}H_{19}N_4O_3$) C, H, N.

Ethyl (*R,S*)-4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (10). To a solution of **9** (200 mg, 0.6 mmol) in a mixture of DMF (2 mL), MeOH (30 mL) and $CHCl_3$ (70 mL) was added 5% palladium on activated carbon (600 mg) and the suspension was hydrogenated in a Parr apparatus at room temperature and 45 psi hydrogen pressure for 5.5 h. TLC showed the disappearance of starting material (R_f 0.60 and 0.58) and the formation of one major spot at 0.57 and one minor spot at 0.55 ($MeOH/CHCl_3$, 1:5). The reaction mixture was filtered through Celite and the residue was washed with 20% DMF in methanol 100 mL. The filtrate was evaporated to dryness under reduced pressure and the solid was dissolved in 100 mL methanol. To this solution was added 3 g of silica gel and the solvent was evaporated to dryness under reduced pressure. This silica gel plug was loaded on a dry silica gel column (2 × 15 cm) and flash chromatographed initially with $CHCl_3$ (200 mL), then sequentially with 200 mL 2% MeOH in $CHCl_3$, 200 mL 4% and 6% MeOH in $CHCl_3$. Fractions which showed the major spot at R_f 0.57 were pooled and evaporated to dryness and recrystallized from ethyl ether and MeOH to afford 150 mg (75%) of **10** as yellow needles: mp 219.7−222 °C; ^{1}H NMR (DMSO-d_6) δ 1. 25−1.32 (m, 6 H, C9−CH_3, OCH_2CH_3), 2.95 (br, 2 H, C8−CH_2), 3.03−3. 15 (m, 1 H, C9−CH), 4.27−4.29 (q, 2H, OCH_2CH_3), 5.99 (s, 2 H, 4-NH_2), 6.47 (s, 2 H, 2-NH_2), 6.88 (s, 1 H, C6−CH), 7.36−7.38 (d, 2 H, 3′-, 5′-CH), 7.84−7.86 (d, 2 H, 2′-, 6′-CH). Anal. ($C_{18}H_{21}N_4O_3$) C, H, N.

(*R,S*)-4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoic Acid (11). To a solution of **10** (150 mg, 0.4 mmol) in MeOH/DMSO (2:1, 22 mL) was added 1 N NaOH (6 mL) and the mixture was stirred under N_2 at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f 0.57) and formation of one major spot at the origin ($MeOH/CHCl_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in distilled water (6 mL) and the solution was filtered through Celite and the Celite pad was washed with 4 mL water. The filtrate was cooled in an ice bath and the pH was adjusted to 4 by dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice−acetone bath and thawed in the refrigerator to 4−5 °C and filtered. The residue was washed with a small amount of cold water and ethyl acetate and dried in vacuo using P_2O_5 to afford 125 mg (90%) of **11** as a white powder: mp 266.6−268 °C; TLC R_f 0.32 ($MeOH/CHCl_3$, 1:5); ^{1}H NMR (DMSO-d_6) δ 1.25−1. 32 (d, 6 H, C9−CH_3), 2.90−3.10 (br, 3 H, C8−CH_2 and C9−CH), 5.97 (s, 2 H, 4-NH_2), 6.41 (s, 2 H, 2-NH_2), 6.88 (s, 1 H, C6−CH), 7.12−7.15 (d, 2 H, 3′-, 5′-CH), 7.73−7.76 (d, 2 H, 2-′, 6′-CH). Anal. ($C_{16}H_{16}N_4O_3 \cdot 0.75H_2O \cdot 1.0HCl$) C, H, N.

(*R,S*)-*N*-[4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzyl]-L-glutamic Acid Diethyl Ester (12). To a solution of **11** (120 mg, 0.37 mmol) in anhydrous DMF (9 mL) was added triethylamine (130 μL) and the mixture stirred under nitrogen at room temperature for 5 min. The resulting solution was cooled to 0 °C, isobutyl chloroformate (130 μL 0.96 mmol) was added and the mixture was stirred at 0 °C for 30 min. At this time TLC ($MeOH/CHCl_3$, 1:5) indicated the formation of activated intermediate at R_f 0.53 and the disappearance of the starting acid R_f 0.32. Diethyl L-glutamate hydrochloride (240 mg, 0.96 mmol) was added to the reaction mixture followed immediately by triethylamine (130 μL, 0.96 mmol). The reaction mixture was slowly allowed to warm to room temperature and stirred under nitrogen for 6 h. The reaction mixture was then subjected to another cycle of activation and coupling using half the quantities listed above. The reaction mixture was slowly allowed to warm to room temperature and stirred under nitrogen for 24 h. The reaction mixture was then subject to third round of activation and coupling using the same the quantities as second round and was stirred for additional 24 h. TLC showed the formation of one major spot at R_f 0.54 ($MeOH/CHCl_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in minimum amount of CH_3Cl/MeOH, 4:1, and chromatographed on a silica gel column (2× 15 cm) and with 4% MeOH in $CHCl_3$ as the eluent. Fractions which showed the desired single spot at R_f 0.54 were pooled and evaporated to dryness and crystallized from ethyl ether and MeOH to afford 120 mg (75%) of **12** as a yellow solid: mp 118.8−120.9 °C; ^{1}H NMR (DMSO-d_6) δ 1.10−1.26 (m, 8 H, C9−CH_3, 2× OCH_2CH_3), 1.96−2.16 (m, 2 H, −$CHCH_2CH_2CO$−), 2.32−2.44 (m, 2 H, −$CHCH_2CH_2CO$−), 2.95−3.05 (br, 2 H, C8−CH_2), 3.06−3.15 (m, 1 H, C9−CH), 4.00−4.40 (q, 4 H, 2× OCH_2CH_3), 4.41 (m, 1 H, −$NHCH(CH_2)$−), 5.95 (s, 2 H, 4-NH_2), 6.44 (s, 2 H, 2-NH_2), 6.87 (s, 1 H, C6−CH), 7.30−7.33 (d, 2 H, 3′-, 5′-CH), 7.74−7.77 (d, 2 H, 2′-, 6′-CH), 8.61−8.63 (d, 1 H, −CONH−). Anal. ($C_{25}H_{31}N_5O_6$) C, H, N.

(*R,S*)-*N*-[4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzyl]-L-glutamic Acid (5). To a solution of the diester **12** (110 mg, 0.2 mmol) in MeOH (10 mL) was added 1 N NaOH (6 mL) and the mixture was stirred under nitrogen at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f 0.54) and formation of one major spot at the origin ($MeOH/CHCl_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mL), the resulting solution cooled in an ice bath and the pH adjusted to 3−4 with dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice−acetone bath and thawed in the refrigerator to 4−5 °C and filtered. The residue was washed with a small amount of cold water and ethyl acetate and dried in vacuo using P_2O_5 to afford 100 mg (95%) of **5** as a white powder: mp 162.8−165 °C; TLC R_f 0.75 ($MeOH-CHCl_3-NH_3OH$, 3:7:2); ^{1}H NMR (DMSO-d_6) δ 1.25−1.27 (d, 3H, C9−CH_3), 1.94−2.08 (2 sets of t, 2 H, Glu β-CH_2), 2.31−2.36 (t, 2 H, Glu γ-CH_2), 2.96−2.98 (d, 2 H, C8−CH_2), 3.03−3.07 (m, 1 H, C9−CH), 4.37 (m, 1 H, Glu α-CH), 5.96 (s, 2 H, 4-NH_2), 6.44 (s, 2 H, 2-NH_2), 6.87 (s, 1 H, C6−CH), 7.30−7.33 (d, 2 H, 3′-, 5′-CH), 7.75−7.78 (d, 2 H, 2′-, 6′-CH), 8.49−8.52 (d, 1 H, −CONH−), 12.34 (br, 2 H, 2× COOH). Anal. ($C_{21}H_{23}N_5O_6 \cdot 1.5H_2O$) C, H, N.

Synthesis of (*E*)- and (*Z*)-4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl) ethenyl]benzoic Acid (13). To a solution of **9** (150 mg, 0.4 mmol) in MeOH/DMSO (2:1, 22 mL) was added 1 N NaOH (6 mL) and the mixture stirred under nitrogen at room temperature for 16 h. TLC showed the disappearance of the starting material and formation of one major spot at the origin ($MeOH/CHCl_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (6 mL), the solution was

filtered through Celite and the Celite pad was wash with 4 mL water. The filtrate was cooled in an ice bath and the pH adjusted to 4 by dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice−acetone bath and thawed in the refrigerator to 4−5 °C and filtered. The residue was washed with a small amount of cold water and ethyl acetate and dried in vacuo using P_2O_5 to afford of 135 mg (97%) of **13** as a yellow solid: mp >300 °C dec; TLC R_f 0.34 (MeOH/$CHCl_3$, 1:5); ^{1}H NMR (DMSO-d_6) *E*-isomer δ 2.24 (s, 3 H, 9-CH_3), 6.10 (s, 2 H, 4-NH_2), 6.55 (s, 2 H, 2-NH_2), 6.99 (s, 1 H, 8-CH), 7.51 (s, 1 H, 6-CH), 7.75−7.78 (d, 2 H, 3′-, 5′-CH), 7.92−7.95 (d, 2 H, 2′-, 6′-CH),12.7 (br, 1 H, COOH); *Z*-isomer δ 2.19 (s, 3 H, 9-CH_3), 6.04 (s, 2 H, 4-NH_2), 6.32 (s, 1 H, 8-CH), 6.55 (s, 2 H, 2-NH_2), 6.66(s, 1 H, 6-CH), 7.30−7.33 (d, 2 H, 3′-, 5′-CH), 7.86−7.89 (d, 2 H, 2′-, 6′-CH), 12.7 (br, 1 H, COOH). Anal. ($C_{16}H_{14}N_4O_3$·0.4H_2O) C, H, N.

Synthesis of (*E*)- and (*Z*)-*N*-[4-[1-Methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethenyl]benzoyl]-L-glutamic Acid (6 and 7). To a solution of **13** (180 mg, 0.37 mmol) in a anhydrous DMF (9 mL) was added triethylamine (130 μL) and the mixture stirred under nitrogen at room temperature for 5 min. The resulting solution was cooled to 0 °C, isobutyl chloroformate (130 μL 0.96 mmol) was added and the mixture was stirred at 0 °C for 30 min. At this time TLC (MeOH/$CHCl_3$, 1:5) indicated the formation of activated intermediate at R_f 0.61 and the disappearance of the starting acid R_f 0.34. Diethyl L-glutamate hydrochloride (240 mg, 0.96 mmol) was added to the reaction mixture followed immediately by triethylamine (130 μL, 0.96 mmol). The reaction mixture was slowly allowed to warm to room temperature and stirred under nitrogen for 6 h. The reaction mixture was then subjected to another cycle of activation using half the quantities listed above. The reaction mixture was stirred under nitrogen at room temperature overnight. After 24 h, The reaction mixture was subjected to third round of activation using the same the quantities as the second round and was stirred for another 24 h. TLC showed the formation of two major spots centered at R_f 0.59 (MeOH/$CHCl_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in the minimum amount of CH_3Cl/MeOH, 4:1, and chromatographed on a silica gel column (2 × 15 cm) with 4% MeOH in $CHCl_3$ as eluent. Fractions which showed the spot at R_f 0.61−0.58 were pooled and evaporated to dryness and crystallized from ethyl acetate/methanol to afford 120 mg (55%) of **14** as yellowish crystal as the single *E*-isomer: mp 180.8−181.5 °C; R_f 0.61 (MeOH/$CHCl_3$, 1:5); ^{1}H NMR (DMSO-d_6) δ 1.14−1.20 (m, 6 H, −$COOCH_2CH_3$ ×2), 2.13 (m, 2 H, CH−CH_2−CH_2−), 2.24 (s, 3 H, 9-CH_3), 2.47−2.49 (t, 2 H, −CH_2CH_2CO−), 4.01−4.15 (m, 4 H, −$COOCH_2CH_3$ ×2), 4.46 (m, 1 H, −CONH−CH−), 6.09 (s, 2 H, 4-NH_2), 6.52 (s, 2 H, 2-NH_2), 6.97 (s, 1 H, 8-CH), 7.50 (s, 1 H, 6-CH), 7.73−7.76 (d, 2 H, 3′-, 5′-CH), 7.87−7.90 (d, 2 H, 2′-, 6′-CH), 8.76 (d, 1 H, −NHCO−). Anal. ($C_{25}H_{29}N_5O_6$) C, H, N.

To a solution of the diester **14** (110 mg, 0.2 mmol) in MeOH (10 mL) was added 1 N NaOH (6 mL) and the mixture was stirred under nitrogen at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f 0.61) and the formation of one major spot at the origin (MeOH/$CHCl_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mL) and the resulting solution was cooled in an ice bath and the pH adjusted to 3−4 by dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice−acetone bath and thawed in the refrigerator to 4−5 °C and filtered. The residue was washed with a small amount of cold water and ethyl acetate and dried in vacuo using P_2O_5 to afford 100 mg (95%) of **6** as an off-white powder: mp 188.3−191.3 °C; ^{1}H NMR (DMSO-d_6) δ 1.97−2.13 (two sets of triplets, 2 H, Glu β-CH_2), 2.25 (s, 3 H, 9-CH_3), 2.26−2.39 (t, 2 H, Glu γ-CH_2), 4.42 (m, 1 H, Glu α-CH), 6.10 (s, 2 H, 4-NH_2), 6.92 (s, 1 H, 8-CH), 7.50 (s, 1H, 6-CH), 7.74−7.77 (d, 2 H, 3′-, 5′-CH), 7.91−7.94 (d, 2 H, 2′-, 6′-CH), 8.24 (br, 2 H, 2-NH_2), 8.58 (d, 1 H, −NHCO−), 12.7 (br, 2 H, COOH). Anal. ($C_{21}H_{21}N_5O_6$·1.0HCl·0.5H_2O) C, H, N.

The filtrate from the diester **14** was found to contain a majority of the diester **15**, R_f 0.58 (MeOH/$CHCl_3$, 1:5), and was directly used for the next step. Thus to **15** (100 mg, 0.2 mmol) dissolved in MeOH (10 mL) was added 1 N NaOH (6 mL) and the mixture was stirred under nitrogen at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f 0.58) and formation of one major spot at the origin (MeOH/$CHCl_3$, 1:5). The reaction mixture evaporated to dryness under reduced pressure. The residue was dissolved in distilled water (10 mL) and the resulting solution was cooled in an ice bath and the pH adjusted to 2−3 by dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice−acetone bath, thawed in the refrigerator to 4−5 °C and filtered. The residue was washed with a small amount of cold water and ethyl acetate and dried in vacuo using P_2O_5 to afford 90 mg (95%) of **7** (*E*/*Z* 1:4 mixture) as a yellow solid: mp 188.3−198.3 °C; ^{1}H NMR (DMSO-d_6) *E*-isomer δ 1.97−2.13 (two sets of triplets,2 H, Glu β-CH_2), 2.25 (s, 3 H, 9-CH_3), 2.26−2.39 (t, 2 H, Glu γ-CH_2), 4.42 (m, 1 H, Glu α-CH), 6.10 (s, 2 H, 4-NH_2), 6.92 (s, 1 H, 8-CH), 7.50 (s, 1H, 6-CH), 7.77−7.74 (d, 2 H, 3′-, 5′-CH), 7.91−7.94 (d, 2H, 2′-, 6′-CH), 8.24 (br, 2 H,2-NH_2), 8.58 (d, 1 H, −NHCO−), 12.7 (br, 2 H, COOH); *Z*-isomer δ 2.00 (m, 2 H, Glu β-CH_2), 2.19 (s, 3 H, 9-CH_3),. 2.47−2.49 (t, 2 H, Glu γ-CH_2), 4.46 (m, 1 H, Glu α-CH), 6.02 (s, 2 H, 4-NH_2), 6.34 (s, 1 H, 8-CH), 6.53 (s, 2 H, 2-NH_2), 6.64 (s, 1 H, 6-CH), 7.28−7.30 (d, 2 H, 3′-, 5′-CH), 7.80−7.82 (d, 2 H, 2′-, 6′-CH), 8.35 (d, 1 H, −NHCO−), 12.7 (br, 2H, COOH). Anal. ($C_{21}H_{21}N_5O_6$·1.5HCl·1.5H_2O) C, H, N, Cl.

Dihydrofolate Reductase (DHFR) Assay.[33] All enzymes were assayed spetrophotometrically in a solution containing 50 μM dihydrofolate, 80 μM NADPH, 0.05 M Tris HCl, 0.001 M 2-mercaptoethanol, and 0.001 M EDTA at pH 7.4 and 30 °C. The reaction was initiated with an amount of enzyme yielding a change in O.D. at 340 nm of 0.015/min.

Thymidylate Synthase (TS) Assay. TS was assayed spetrophotometrically at 30 °C and pH 7.4 in a mixture containing 0.1 M 2-mercaptoethanol, 0.0003 M (6*R*,*S*)-terahydrofolate, 0.012 M formaldehyde, 0.02 M $MgCl_2$, 0.001 M dUMP, 0.04 M Tris HCl, and 0.00075 M NaEDTA. This was the assay described by Wahba and Friedkin,[34a] except that the dUMP concentration was increased 25-fold according to the method of Davisson et al.[34b] The reaction was initiated by the addition of an amount of enzyme yielding a change in absorbance at 340 nm of 0.016/min in the absence of inhibitor. The percent inhibition was determined at a minimum of four inhibitor concentrations with 20% of the 50% point. The standard deviations for determination of 50% points were within ±10% of the value given.

Cell Lines and Methods for Measuring Growth Inhibitory Potency (Table 3). Solutions used in cell culture studies were standardized using extinction coefficients. Extinction coefficients were determined for **5** (pH 1, λ_{max-1} 249 nm (22900), λ_{max-2} 300 nm (8000); pH 7, λ_{max} 249 nm (22400); pH 13, λ_{max} 249 nm (22500)), for **6** (pH 1, λ_{max} 265 nm (28400); pH 7, λ_{max} 268 nm (30100); pH 13, λ_{max} 268 nm (29700)), and for **7** (pH 1, λ_{max} 250 nm (22600); pH 7, λ_{max} 261 nm (22500); pH 13, λ_{max} 261 nm (22600)). Extinction coefficients for methotrexate (MTX), a generous gift of Immunex (Seattle, WA), were from the literature.[35]

All cell lines were verified to be negative for mycoplasma contamination using the GenProbe test kit. The human T-lymphoblastic leukemia cell line CCRF-CEM[25] and its methotrexate-resistant sublines R1,[26] R2 (Bos),[27] and R30dm[28] used in these studies were cultured as described.[28] R1 expresses 20-fold elevated levels of DHFR, the target enzyme of MTX. R2 has dramatically reduced MTX uptake. R30dm expresses only 1% of the FPGS activity of CCRF-CEM and is resistant to short-term, but not continuous, MTX exposure; however, R30dm is generally cross-resistant in continuous exposure to antifolates requiring polyglutamylation to form potent potent inhibitors. The A253 and FaDu human head and neck squamous cell carcinoma monolayer cells were propagated in RPMI 1640/10% fetal calf serum (FCS); however, growth inhibition was measured in medium containing 5% FCS[36] to minimize

the levels of purine and pyrimidines introduced from FCS. Growth inhibition of all cell lines by continuous drug exposure was assayed as described. EC_{50} values were determined from plots of percent control growth versus logarithm of drug concentration.

Protection against growth inhibition of FaDu cells was assayed by including metabolites simultaneously with a concentration of drug previously determined to inhibit growth by 80−95%; the remainder of assay was as described. Metabolites included were: leucovorin (LV, 0.1−10 μM); or thymidine (TdR, 40 μM); or hypoxanthine (Hx, 50 μM); or a combination of TdR and Hx at the same individual concentrations. Growth inhibition was measured relative to the appropriate metabolite-treated control; metabolites caused <9% growth inhibition in the absence of drug, however.

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Supporting Information Available: In vitro preclinical antitumor screening results of compound **5** from the NCI (Table 2) and two-dimensional proton−proton ROESY spectra of the *E*-isomer precursor ester **14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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