Synthesis of N-[4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid as an Antifolate[1]

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N-[4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid **3** was designed and synthesized to investigate the effect of homologation of a C9-methyl to an ethyl on dihydrofolate reductase (DHFR) inhibition and on antitumor activity. Compound **3** was obtained via a concise seven step synthesis starting from palladium-catalyzed carbonylation of 4-propionylphenol, followed by a Wittig reaction with 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (**6**), catalytic hydrogenation, hydrolysis, and standard peptide coupling with diethyl L-glutamate. The biological results indicated that extending the C9-methyl group to an ethyl on the C8−C9 bridge region (analogue **3**) doubled the inhibitory potency against recombinant human (rh) DHFR (IC_{50} = 0.21 μM) as compared to the C9-methyl analogue **1** and was 4-fold more potent than the C9−H analogue **2**. As compared to **1**, compound **3** demonstrated increased growth inhibitory potency against several human tumor cell lines in culture with GI_{50} values $< 1.0 \times 10^{-8}$ M. Compound **3** was also a weak inhibitor of rh thymidylate synthase. Compounds **1** and **3** were efficient substrates of human folylpolyglutamate synthetase (FPGS). Further evaluation of the cytotoxicity of **3** in methotrexate-resistant CCRF-CEM cell sublines and metabolite protection studies implicated DHFR as the primary intracelluar target. Thus, alkylation of the C9 position in the C8−C9 bridge of the classical 5-substituted 2,4-diaminofuro-[2,3-*d*]pyrimidine is highly conducive to DHFR and tumor inhibitory activity as well as FPGS substrate efficiency.

Introduction

Folate metabolism is an attractive target for cancer chemotherapy, due to its crucial role in the biosynthesis of nucleic acid precursors.[2] Inhibition of folate-dependent enzymes in cancer cells, microbial cells, and in protozoan cells provides compounds that have found clinical utility as antitumor, antimicrobial, and antiprotozoal agents.[3,4]

As part of a continuing effort in our laboratory to develop novel classical antifolates as dihydrofolate reductase (DHFR) inhibitors and as antitumor agents, Gangjee et al.[5,6] reported the synthesis of N-[4-[1-methyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl-L-glutamic acid (**1**) and its C9−H analogue **2** (Figure 1). The C9-methyl analogue **1** was twice as inhibitory against recombinant human (rh) DHFR and about 10 times greater in its growth inhibitory potency against tumor cells in culture (CCRF-CEM EC_{50} 29.2 nM, FaDu EC_{50} 17.5 nM, and A253 EC_{50} 28.3 nM) as compared to **2**. The increased potency against DHFR and against the growth of tumor cells in culture of **1** was, in part, attributed to increased hydrophobic interaction of the C9-methyl moiety with DHFR and by its increased lipophilicity. Molecular modeling studies using Sybyl 6.5[7] suggested that extending the C9-methyl to an ethyl moiety could further enhance both the hydrophobic interaction with the enzyme and increase the lipophilicity, which might provide more potent inhibition of the growth of tumor cells. Similar homologation of a methyl moiety to an ethyl in the C9−C10 bridge region of 6−6 fused bicylic analogues derived from deaza aminopterin (AMT) has been extensively studied[8−11] and led to the discovery of edatrexate,[10] the 10-ethyl-10-deaza AMT analogue, which was advanced to phase III clinical trials. DeGraw et al.[11a,b] reported that in the 10-alkyl-5,10-dideaza AMT analogues, homologation of the 10-methyl moiety to an ethyl increased its growth inhibitory potency against tumor cells in culture and, more importantly, alkylation of the

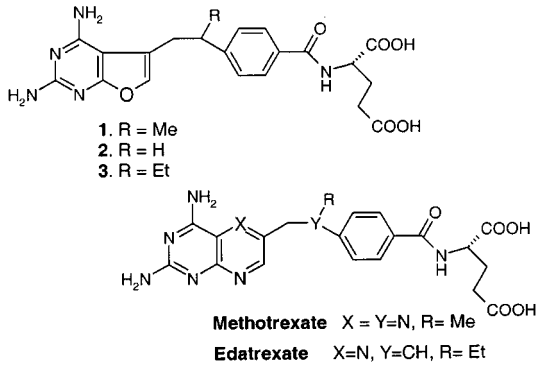

Figure 1.

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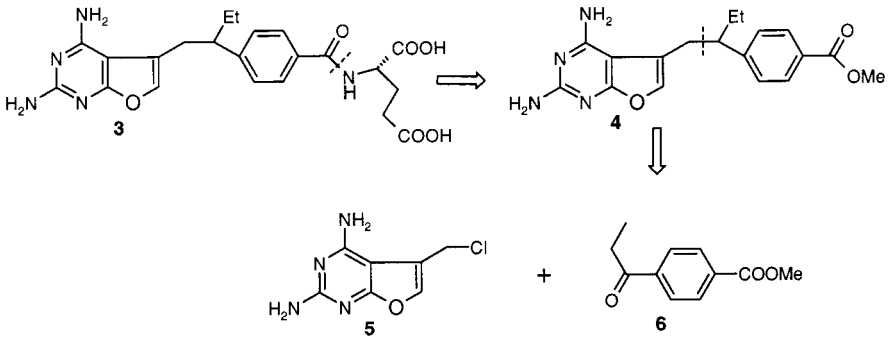
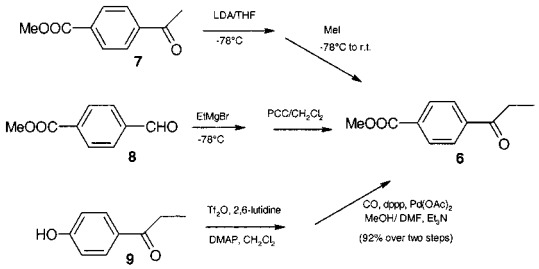
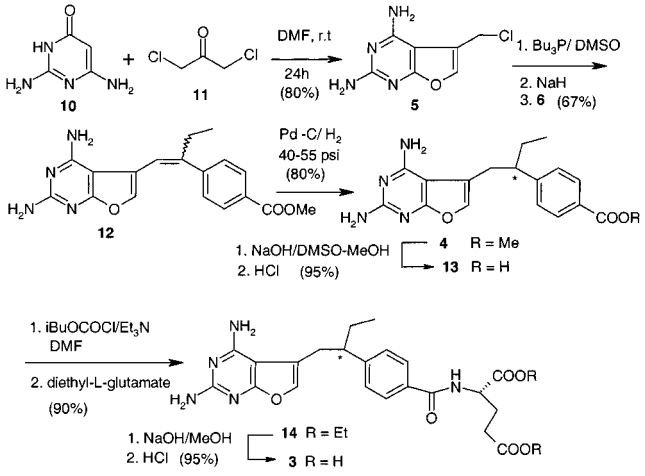


Figure 2.

Scheme 1



Scheme 2



bridge C10 carbon enhanced transport of the antifolate into tumor cells. As part of a structure−activity relationship study on the C8−C9 bridge region of classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine antifolates, we designed and synthesized N-[4-[1-ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid **3**, the 9-ethyl homologue of **1**, to investigate the effect of a C9-ethyl on the inhibitory activity against DHFR and against the growth of tumor cells in culture.

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 the binding affinity for folate-dependent enzymes.[12a,b] Alkylation on the bridge carbon C10 of the 10-deaza AMT analogue was reported to enhance the substrate efficiency of the analogue for FPGS.[13] It was thus of interest to also determine the effect of a C9-ethyl modification with respect to FPGS activity.

In addition, thymidylate synthase (TS), which catalyzes the sole de novo synthesis of deoxythymidylate (dTMP) from deoxyuridine monophosphate (dUMP), is a folate cofactor-requiring enzyme.[14] It was also of interest to evaluate **3** as an inhibitor of TS.

Chemistry

From a retrosynthetic point of view (Figure 2), it was anticipated that a Wittig type condensation of the 5-chloromethyl group of 2,4-diamino-5-(chloromethyl)-furo[2,3-*d*]pyrimidine (**5**) with an appropriately substituted propiophenone **6** would afford the desired 9-ethyl furo[2,3-*d*]pyrimidine nucleus **4** of the target compound. Sequential reduction, hydrolysis, peptide coupling, and saponification would afford the desired target **3**.

Thus, the first step was the synthesis of 4-propionylbenzoic acid ester (**6**). Three possible synthetic approaches are shown in Scheme 1. The first attempt employed enolate chemistry starting from commercially available 4-acetylbenzoic acid ester **7**. The enolate was generated in situ using LDA at −78 °C and subsequent reaction with MeI. The desired product was difficult to separate from the starting methyl ketone. Repeated recrytallization afforded the pure ethyl ketone **6** in only 40% isolated yield.

The second method was an alkylation reaction, using the appropriate Grignard reagent, of methyl 4-formylbenzoate (**8**) followed by a PCC oxidation. Though the secondary alcohol was isolated in 90% yield, the PCC oxidation resulted in degradation of the methyl ester and the pure ethyl ketone **6** was isolated in only 45% yield. The final strategy employed a modified Gerlach's palladium-catalyzed carbonylation[15] from the 4-propionylphenol triflate. Thus, 4-propionylphenol triflate was obtained in quantitative yield from the commercially available 4-hydroxypropiophenone **9** (Scheme 1) and was directly subjected to palladium-catalyzed carbonylation using dppp/$Pd(OAc)_2$ (1/1.3 ratio) as catalyst (5−6% equivalent to that of triflate) and dimethyl formamide (DMF)/MeOH (4/3) as solvent and afforded the desired ethyl ketone **6** in 92% yield over two steps.

With the desired ethyl ketone **6** in hand, the next step was the condensation with intermediate **5** (Scheme 2), which in turn was obtained from 2,6-diaminopyrimidin-4-one **10** and 1,3-dichloroacetone **11**.[6,16] Wittig condensation of **5** and **6** in dimethyl sulfoxide (DMSO), using excess *n*-Bu_3P and NaH to form the ylide and methanol to quench the reaction,[5] furnished the olefin **12** in 67% yield as a mixture of *E* and *Z* isomers. Catalytic hydrogenation of **12** under optimized conditions followed

Table 1. Inhibitory Concentration (IC_{50} in μM) against Isolated DHFR and TS[a]

	DHFR			TS	
	rh	*E. coli*	*T. gondii*	rh	*E. coli*
1[b]	0.42	1.1	2.1	>360	100
2[c]	1.0	ND	ND	220	ND
3	0.22	0.44	1.1	220	220
MTX	0.022	0.009	0.011	ND	ND
PDDF	ND	ND	ND	0.15	0.1

[a] 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. [b] Data derived from ref 5. [c] Data derived from ref 6; ND = not determined.

Table 2. Activity of **3** as a Substrate for rh FPGS[a]

substrate	K_m (μM)	V_{max} (rel)	V_{max}/K_m	n
AMT	3.2 ± 0.3	1	0.31 ± 0.03	3
3	1.1 ± 0.1	0.37 ± 0.01	0.34 ± 0.05	2
1[b]	1.2 ± 0.3	0.52 ± 0.03	0.46 ± 0.09	2
2[c]	8.5 ± 2.1	0.65 ± 0.01	0.07 ± 0.02	2

[a] FPGS substrate activity was determined as described in ref 6. Values presented are average ± SD if $n \geq 3$ and average ± range for $n = 2$. V_{max} is calculated relative to AMT within the same experiment. [b] Data from ref 5. [c] Data from ref 6.

by column chromatography and recrystallization from MeOH afforded **4** in 80% yield. Saponification of **4** with aqueous sodium hydroxide gave the free acid **13** in 95% yield. Subsequent coupling of **13** with diethyl L-glutamate using isobutyl chloroformate as the coupling reagent afforded the product **14** in 90% yield. Final saponification with aqueous sodium hydroxide at room temperature followed by acidification to pH 4 in an ice bath afforded the diacid **3** in 95% yield. The structures of **3** and all intermediates were characterized by ^{1}H nuclear magnetic resonance (NMR) and elemental analysis.

Biological Evaluation and Discussion

Compound **3** was evaluated as an inhibitor of *Escherichia coli* (ec), *Toxoplasma gondii* (tg), and rh DHFR.[17−19] The inhibitory potency (IC_{50}) values are compared with methotrexate (MTX) in Table 1 and the previously reported values for **1** and **2**.[5,6] The inhibitory potency of **3** is twice that of its C9-methyl analogue **1** and four times that of analogue **2**. Against ecDHFR and tgDHFR, compound **3** was also twice as potent as the C9-methyl, **1**.

Analogue **3** was also evaluated as an inhibitor of ecTS and rhTS[20,21] and was a marginal inhibitor of these enzymes with IC_{50} values ≥ 200 μM in most cases (Table 1).

Because polyglutamylation plays a role in the mechanism of action of some classical antifolates,[12a,b] it was of interest to evaluate analogue **3** as a substrate for human FPGS (Table 2). The data indicate that the 9-ethyl analogue **3** is as efficient as AMT as a FPGS substrate; both **3** and **1** also have a 7-fold lower K_m and greater than 5-fold higher substrate efficiency relative to the unalkylated parent **2**.[5] This result was consistent with those obtained for 10-deaza AMT analogues, in which alkylation of the C10 by an ethyl increased its FPGS substrate efficiency as compared to the C10 unalkylated analogue.[13] The results obtained with **1** and **3** suggest that alkylation of the C9 of classical 5-substituted furo[2,3-*d*]pyrimidines is highly beneficial with regard to FPGS substrate activity. These data further suggest that polyglutamylation may be important in the mechanism of action of these novel furo[2,3-*d*]pyrimidines. Because **3** 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.

The target compound **3** was also evaluated as an inhibitor of the growth of CCRF-CEM human leukemia cells in culture during continuous exposure (Table 3).[22−24] Compound **3** was highly cytotoxic with an EC_{50} of 35.5 nM, which is similar to that of MTX and is 8-fold more potent than **2** and equipotent with **1**. This indicates that C9-alkylation (as in **1** and **3**) is also highly conducive to tumor growth inhibition in culture.

Because compound **3** was 10-fold less potent than MTX against isolated rhDHFR but only about 2-fold less potent against the growth of tumor cells (CCRF-CEM) in culture, it was of interest to further define its mechanism of action. Compound **3** was thus evaluated as an inhibitor of the growth of three CCRF-CEM sublines with defined mechanisms of resistance to MTX.[22−26] Against the increased DHFR subline R1, compound **3** was about 80-fold less potent than against parent CCRF-CEM. Thus, it was quantitatively similar to MTX (72-fold). These data are consistent with DHFR being the primary target of analogue **3**. Cross-resistance of the MTX transport deficient subline R2 to **3** indicated that it uses the MTX/reduced folate carrier (RFC) for uptake. However, the degree of cross-resistance to **3** (25-fold) was much less than to MTX (180-fold), which may suggest that the mutation in the MTX/RFC affects transport of 2,4-diaminofuro[2,3-*d*]pyrimidines to a lesser extent 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 **3** is as poorly transported as is MTX but it is more readily converted to longer ($Glu_{\geq 3}$), more potent polyglutamate inhibitors and that this more efficient metabolism compensates for the poor transport. The kinetic constant for monoglutamyl **3** with human FPGS (which primarily measures conversion to the diglutamate) has been determined (Table 2). The data show that **3** is a much more efficient FPGS substrate than is MTX.

Interestingly, compound **3** showed only a slight decrease in potency against the FPGS deficient cell line R30dm, which suggests that either the polyglutamates are not required for the antitumor activity of **3** in continuous exposure or the polyglutamates are formed very efficiently. These two possibilities are currently being explored. However, on the basis of the data in Table 2 showing that analogue **3** is a good substrate for FPGS, it is more likely that the later possibility operates. Although DHFR is often insensitive to the polyglutamylation status of substrates and inhibitors,[27] enhanced potency of polyglutamates of antifolates against DHFR has been reported.[28a,b]

Metabolite protection studies were carried out with **3** in order to further elucidate its mechanism of action. Both **3** (at 100 or 200 nM) and MTX (at 40 and 50 nM) drug levels that inhibited growth by 95% were fully

Table 3. Growth Inhibition of CCRF-CEM Human Leukemia Cells, Its MTX-Resistant Sublines with Defined Mechanism during Continuous Exposure (0−120 h) EC_{50} in nM[a]

cell lines	MTX	**1**[b]	**2**[c]	**3**
CCRF-CEM	14.5 ± 0.5 (n = 4)	29.2 ± 0.5 (n = 5)	290 ± 10 (n = 3)	35.5 ± 1.5 (n = 2)
R30dm	15.0 ± 0 (n = 2)	220 ± 20 (n = 2)	4250 ± 50 (n = 2)	50 ± 8 (n = 2)
R1	1050 ± 50 (n = 2)	685 ± 45 (n = 2)	ND	2100 ± 100 (n = 2)
R2	2600 ± 100 (n = 2)	430 ± 60 (n = 2)	ND	890 ± 110 (n = 2)

[a] EC_{50} = concentration of drug required to decrease cell growth by 50% after 5 days of treatment. [b] Data from ref 5. [c] Data from ref 6; ND = not determined.

Table 4. Cytotoxicity Evaluation (GI_{50}, M) of Compound **3**[a] Against Selected Tumor Cell Lines as Compared to Compound **1**[29]

cell lines	compd **3**	compd **1**[5]	cell lines	compd **3**	compd **1**[5]
	Ovarian Cancer			Melanoma	
IGROV1	$<1.0 \times 10^{-8}$	7.14×10^{-8}	LOX IMVI	2.37×10^{-8}	$<1.0 \times 10^{-8}$
OVCAR-5	$<1.0 \times 10^{-8}$	6.68×10^{-8}	SK-MEL-5	8.18×10^{-8}	7.22×10^{-7}
	Nonsmall Cell Lung Cancer			Colon Cancer	
A549/ATCC	1.38×10^{-8}	5.81×10^{-8}	HCT-15	$<1.0 \times 10^{-8}$	7.39×10^{-8}
NCI-H460	$<1.0 \times 10^{-8}$	1.73×10^{-8}	HT29	2.39×10^{-8}	$<1.0 \times 10^{-8}$
NCI-H522	$<1.0 \times 10^{-8}$	ND	SW-620	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
	CNS Cancer			Renal Cancer	
SF-268	$<1.0 \times 10^{-8}$	6.13×10^{-8}	ACHN	$<1.0 \times 10^{-8}$	1.43×10^{-8}
SF-539	$<1.0 \times 10^{-8}$	8.78×10^{-8}	CAKI-1	$<1.0 \times 10^{-8}$	ND
SF-295	$<1.0 \times 10^{-8}$	8.94×10^{-7}	TK-10	3.40×10^{-7}	$>1.0 \times 10^{-4}$
U251	2.32×10^{-8}	2.32×10^{-8}	UO-31	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
	Leukemia			Breast Cancer	
CCRF-CEM	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$	MCF7	$<1.0 \times 10^{-8}$	2.70×10^{-8}
HL-60 (TB)	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$	MDA-MB-435	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
K-562	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$	MDA-N	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
RPMI-8226	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$			
SR	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$			

[a] Sensitivity of the other tumor cell lines in the NCI panel to **3**: GI_{50} values $> 1.0 \times 10^{-4}$ M are SK-MEL-2, EKVX, NCI-H226, RXF 393, MDA-MB-231/ATCC, HS 578T, BT-549, T-47D, and SNB-75; GI_{50} values in 10^{-5} to 10^{-6} M is H-23; GI_{50} values in 10^{-6} to 10^{-7} M are HOP-62 and HOP-92; GI_{50} values in 10^{-7} to 10^{-8} M are NCI/ADR-RES, SN12C, and HCT-116; GI_{50} values in 10^{-8} M are UACC-257, UACC-62, and SK-OV-3; GI_{50} values $< 1.0 \times 10^{-8}$ M are OVCAR-8, UO-31, and 786-0.

protected by as little as 0.1 μM leucovorin indicating that **3** acts as an antifolate (data not shown). These results are consistent with DHFR being the primary intracellular target of **3** (although other targets are not excluded) and are also consistent with the cross-resistance studies (Table 3) and with previous data on the classical furo[2,3-*d*]pyrimidine antifolates **1** and **2**.[5,6]

Compound **3** was selected by the National Cancer Institute (NCI)[29] for evaluation in its in vitro preclinical antitumor screening program. The ability of compound **3** to inhibit the growth of tumor cells 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 **3** showed GI_{50} values in the 10^{-8} M range or less. It is noteworthy that in a variety of tumor cell lines (Table 4), compound **3** had more than 5-fold increased inhibitory potency as compared to the C9-methyl analogue **1**. This increased potency in continuous exposure may reflect the greater inhibitory potency of the C9-ethyl analogue against human DHFR (Table 1), although differences in DHFR sensitivity, drug transport, and intracellular polyglutamate accumulation may also contribute in various cell lines. It was also interesting to note that (data not shown), like compound **1**, compound **3** 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. These data also support the previous hypothesis that alkylation of the bridge carbon of the classical deaza folate analogues could enhance the transport into certain tumor cells.[11,30] This compound is currently under further evaluation by the NCI as an antitumor agent.

In summary, C9-ethyl classical 5-substituted, 2,4-diaminofuro[2,3-*d*]pyrimidine antifolate **3** was designed and synthesized, where the C9-methyl was homologated to an ethyl. The biological results show that extending the C9-methyl group to an ethyl in the C8−C9 bridge (analogue **3**) increases DHFR inhibitory activity as compared to the C9-methyl analogue **1** and the C9-unalkylated analogue **2**. As compared to compound **1**, extending the C9-methyl to an ethyl also results in increased inhibitory potency against the growth of several tumor cell lines in culture. Both **1** and **3** are more efficient as FPGS substrates than **2**. Compounds **1** and **3** are also more potent than **2** (>8-fold) as inhibitors against the growth of CCRF-CEM leukemia cells in culture. These data show that the alkylation of the C9 position in the C8−C9 bridge of classical 5-substituted 2,4-diaminofuro[2,3-*d*]pyrimidine is highly conducive to DHFR and tumor inhibitory activity as well as FPGS substrate efficiency.

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mm Hg) 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. NMR spectra for proton (^{1}H) 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 = multiplet, br = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. 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 illumina-

tion. 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.

Methyl 4-Propionylbenzoate (6). A 7.5 g (50 mmol) amount of 4-hydroxy propiophenone (**9**) was suspended in 300 mL of anhydrous CH_2Cl_2 and cooled to −30 °C. A 6.42 g (60 mmol) amount of 2,6-lutidine, 1.22 g (10 mmol) of 4-*N*,*N*-dimethylaminopyridine, and 17 g (60 mmol) of trifluoromethanesulfonic acid anhydride were added. The resulting mixture was stirred under N_2 at ambient temperature for 2 h and washed with 2 × 50 mL saturated NH_4Cl solution and 2 × 100 mL of 1 N HCl. The organic layer obtained was dried under anhydrous Na_2SO_4 and after evaporation of the solvent under reduced pressure afforded 14 g of 4-propionylphenol trifluoromethanesulfonate as a yellowish oil, which was used directly for the next step.

A mixture of 4-propionylphenol trifluoromethanesulfonate (14 g, 50 mmol) and triethylamine (25 mL, 180 mmol) in 150 mL of DMF was charged with palladium acetate (0.9 g, 4 mmol), 1,3-bis(diphenylphosphino)propane (1.4 g, 3 mmol), and 110 mL of anhydrous methanol. The resulting suspension was purged with carbon monoxide for 5 min and then stirred under carbon monoxide for 4 h at 70 °C. A 300 mL amount of water was added to the reaction mixture, and the resulting mixture was extracted with 3 × 200 mL ethyl acetate and dried under Na_2SO_4. After evaporation and column chromatography (4 × 20 cm) on silica gel (Hexane/EtOAc, 2:1), the mixture afforded 8.9 g (92%, lit.[12] 80%) of methyl 4-propinoylbenzote as white crystals; mp 80−81.5 °C (lit.[31] 80−81 °C). ^{1}H NMR ($CDCl_3$): δ 1.21−1.25 (t, 3 H, $-COCH_2CH_3$), 2.99−3.07 (q, 2 H, $-COCH_2CH_3$), 3.94 (s, 3 H, OMe), 7.99−8.02 (d, 2 H, Ph-CH), 8.09−8.12 (d, 2 H, Ph-CH); Anal. ($C_{11}H_{12}O_3$) C, H.

Methyl (*E*/*Z*)-4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethenyl]benzoate (12). To a solution of 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine **6**[6,16] (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 methyl 4-propionylbenzoate (1.2 g, 5.5 mmol). The reaction mixture was stirred at room temperature for 38 h. TLC showed the disappearance of the starting material (R_f 0.45) and appearance of two spots at 0.73 and 0.78 (MeOH/$CHCl_3$, 1:5). The reaction was quenched with MeOH (80 mL). The resulting solution was evaporated, and 100 mL of methanol was added to dissolve the residue, followed by 10 g of 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) and then sequentially with 300 mL of 2−5% MeOH in $CHCl_3$. Fractions that showed the major spot at R_f 0.73 along with the spot at R_f 0.78 were pooled and evaporated to dryness. Recrystallization from ethyl acetate afforded 1.15 g (67%) of **12** (*Z*:*E* ratio 1:1) as yellow needles; mp 189.9−193.4 °C. ^{1}H NMR (DMSO-d_6): *E*-isomer: δ 0.95−1.00 (t, 3 H, C9−CH_2CH_3), 2.50−2.55 (q, 2 H, C9−CH_2CH_3), 3.86 (s, 3 H, OMe), 6.09 (s, 2 H, 4- or 2-NH_2), 6.47 (s, 2 H, 2- or 4-NH_2), 6.86 (s, 1 H, C8−CH), 7.44 (s, 1 H, C6−CH), 7.71−7.74 (d, 2 H, Ph-CH), 7.94−7.97 (d, 2 H, Ph-CH); *Z*-isomer: δ 0.95−1.00 (t, 3 H, C9−CH_2CH_3), 2.69−2.74 (q, 2 H, C9−CH_2CH_3), 3.84 (s, 3 H, OMe), 6.02 (s, 2 H, 4- or 2-NH_2), 6.21 (s, 1 H, C8−CH), 6.50 (s, 2 H, 2- or 4-NH_2), 6.62 (s, 1 H, C6−CH), 7.28−7.31(d, 2 H, Ph-CH), 7.90−7.93 (d, 2 H, Ph-CH). Anal. ($C_{18}H_{18}N_4O_3$·0.3CH_3COOEt) C, H, N.

Methyl (*R*,*S*)-4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (4). To a solution of **12** (175 mg, 0.5 mmol) in a mixture of MeOH (20 mL) and $CHCl_3$ (50 mL) was added 10% palladium on activated carbon (350 mg), and the suspension was hydrogenated in a Parr apparatus at room temperature and 45 psi hydrogen pressure for 8 h. TLC showed the disappearance of starting material (R_f 0.78 and 0.73) and the formation of one major spot at R_f 0.76 (MeOH/$CHCl_3$, 1:5). The reaction mixture was filtered through Celite, and the residue was washed with MeOH/$CHCl_3$ (1:1) 100 mL. The filtrate was evaporated to dryness under reduced pressure, and 100 mL of methanol was added to the solid. 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) and then sequentially with 200 mL of 2% MeOH in $CHCl_3$ and 200 mL of 4 and 6% MeOH in $CHCl_3$. Fractions that showed the major spot at R_f 0.57 were pooled and evaporated to dryness and recrystallized from ethyl ether and MeOH to afford 145 mg (80%) of **4** as white needles; mp 199−201.5 °C. TLC R_f 0.76 (MeOH/$CHCl_3$, 1:5). ^{1}H NMR (DMSO-d_6): δ 0.66−0.71 (t, 3 H, C9−CH_2CH_3), 1.58−1.80 (m, 2 H, C9−CH_2CH_3), 2.87−3.17 (m, 3 H, C9−CH, C8−CH_2), 3.83 (s, 3 H, OMe), 5.93 (s, 2 H, 4- or 2-NH_2), 6.40 (s, 2 H, 2- or 4-NH_2), 6.83 (s, 1 H, C6−CH), 7.31−7.34 (d, 2 H, Ph-CH), 7.81−7.86 (d, 2 H, Ph-CH). Anal. ($C_{18}H_{20}N_4O_3$·0.4 H_2O) C, H, N.

(*R*,*S*)-4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoic Acid (13). To a solution of **4** (130 mg, 0.38 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 0.76) 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), the solution was filtered through Celite, and the Celite pad was washed with 4 mL of 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 120 mg (95%) of **13** as a yellow powder. TLC R_f 0.40 (MeOH/$CHCl_3$, 1:5); mp 256.5−258.3 °C. ^{1}H NMR (DMSO-d_6): δ 0.66−0.71 (t, 3 H, C9−CH_2CH_3), 1.58−1.80 (m, 2 H, C9−CH_2CH_3), 2.87−3.17 (m, 3 H, C9−CH, C8−CH_2), 5.93 (s, 2 H, 4- or 2-NH_2), 6.40 (s, 2 H, 2- or 4-NH_2), 7.09 (s, 1 H, C6−CH), 7.30−7.32 (d, 2 H, Ph-CH), 7.81−7.83 (d, 2 H, Ph-CH), 12.83 (br, 1 H, COOH). Anal. ($C_{17}H_{18}N_4O_3$·0.2 H_2O) C, H, N.

(*R*,*S*)-N-[4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid Diethyl Ester (14). To a solution of **13** (120 mg, 0.37 mmol) in anhydrous DMF (9 mL) was added triethylamine (134 μL), and the mixture was 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 the activated intermediate at R_f 0.75 and the disappearance of the starting acid R_f 0.40. 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 subjected to a third round of activation and coupling using the same quantities as the second round and was stirred for an additional 24 h. TLC showed the formation of one major spot at R_f 0.76 (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) and with 4% MeOH in $CHCl_3$ as the eluent. Fractions that showed the desired spot were pooled and evaporated to dryness and crystallized from ethyl ether and MeOH to afford 170 mg (90%) of **14** as yellow crystals; mp 99.7−102.1 °C. ^{1}H NMR (DMSO-d_6): δ 0.66−0.71 (t, 3 H, C9−CH_2CH_3), 0.93−1.20 (m, 6 H, 2 × OCH_2CH_3), 1.58−1.80 (two sets of m, 2 H, C9−CH_2CH_3), 1.98−2.10 (m, 2 H, −$CHCH_2CH_2CO$-), 2.39−2.45 (m, 2 H, −$CHCH_2CH_2CO$-), 2.87−3.08 (m, 3 H, C9−CH, C8−CH_2), 4.03−4.13 (m, 4 H, 2 × OCH_2CH_3), 4.42 (m, 1 H, −$NHCH(CH_2)$-), 5.94 (s, 2 H, 4- or 2-NH_2), 6.41 (s, 2 H, 2- or 4-NH_2), 6.83 (s, 1 H, C6−CH), 7.27−7.29 (d, 2 H, 3′-, 5′-CH), 7.74−7.76 (d, 2 H, 2′-, 6′-CH), 8.61−8.63 (d, 1 H, −CONH-). Anal. ($C_{26}H_{33}N_5O_6$) C, H, N.

(*R*,*S*)-N-[4-[1-Ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid (3). To a solution of the diester **14** (150 mg, 0.29 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 0.76) 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 was cooled in an ice bath, and the pH was 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 130 mg (95%) of **3** as a white powder; mp 171.3−173.5 °C. TLC R_f 0.80 (MeOH/$CHCl_3$/NH_3-OH, 3:7:2). ^{1}H NMR (DMSO-d_6): δ 0.66−0.71 (t, 3 H, C9−CH_2CH_3), 1.58−1.80 (two sets of m, 2 H, C9−CH_2CH_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), 2.87−3.16 (m, 3 H, C8−CH_2, C9−CH), 4.41 (m, 1 H, Glu α-CH), 6.17 (s, 2 H, 4- or 2-NH_2), 6.66 (s, 2 H, 2- or 4-NH_2), 6.88 (s, 1 H, C6−CH), 7.27−7.29 (d, 2 H, 3′-, 5′-CH), 7.75−7.77 (d, 2 H, 2′-, 6′-CH), 8.52−8.54 (d, 1 H, −CONH-), 12.38−12.53 (br, 2 H, 2 × COOH). Anal. ($C_{22}H_{25}N_5O_6$·$0.5H_2O$) C, H, N.

DHFR Assay.[32] All enzymes were assayed spectrophotometrically 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.

TS Assay. TS was assayed spectrophotometrically at 30 °C and pH 7.4 in a mixture containing 0.1 M 2-mercaptoethanol, 0.0003 M (6*R*,*S*)-tetrahydrofolate, 0.012 M formaldehyde, 0.02 M $MgCl_2$, 0.001 M dUMP, 0.04 M Tris HCl, and 0.000 75 M NaEDTA. This was the assay described by Wahba and Friedkin,[33a] except that the dUMP concentration was increased 25-fold according to the method of Davisson et al.[33b] 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.

Cell Lines and Methods for Measuring Growth Inhibitory Potency. 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[22] and its MTX-resistant sublines R1,[23] R2,[24] and R30dm[25] used in these studies were cultured as described.[25] 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 inhibitors. Growth inhibition of all cell lines by continuous drug exposure was assayed as described.[5,6] EC_{50} values were determined from plots of percent control growth vs logarithm of drug concentration.

Protection against growth inhibition of FaDu cells was assayed by including leucovorin (LV) at 0.1, 1.0, and 10 μM simultaneously with a concentration of drug previously determined to inhibit growth by about 95%; the remainder of the assay was as described.[5,6] Growth inhibition was measured relative to the appropriate LV-treated control; LV caused no significant growth inhibition in the absence of drug.

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