Synthesis and Biological Activity of an Acyclic Analogue of 5,6,7,8-Tetrahydrofolic Acid, *N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-benzoyl]-L-glutamic Acid

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The synthesis and biological evaluation of *N*-[4-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-benzoyl]-L-glutamic acid (1) (5-DACTHF, 543U76), an acyclic analogue of 5,6,7,8-tetrahydrofolic acid (THFA), are described. The key intermediate, hemiaminal 8, was prepared in four stages from 3-chloropropionaldehyde diethyl acetal. Reaction of 8 with dimethyl *N*-(4-aminobenzoyl)-L-glutamate gave the 2,4-bis(acetylamino) derivative 11, which was hydrolyzed with 1 N sodium hydroxide to give 1; the glycine analogue 16 was prepared in a similar manner. The *N*-methyl analogue 2 and *N*-formyl analogue 3 were prepared from 11 and 1, respectively. Compounds 1–3 inhibited growth of Detroit 98 and L cells in cell culture, with IC_{50}s ranging from 2 to 0.018 μM. Cell culture toxicity reversal studies and enzyme inhibition tests showed that 1 was cytotoxic but not by the mechanism of the dihydrofolate reductase inhibitor aminopterin. Compound 1 and its polyglutamylated homologues inhibited glycinamide ribonucleotide transformylase (GAR-TFase) and aminoimidazole ribonucleotide transformylase (AICAR-TFase), the folate-dependent enzymes in de novo purine biosynthesis; and 1 was an effective substrate for mammalian folylpolyglutamate synthetase. The compound inhibited (IC_{50} = 20 nM) the conversion of [^{14}C]formate to [^{14}C]-formylglycinamide ribonucleotide by MOLT-4 cells in culture. These data suggest that the site of action of 1 is inhibition of purine de novo biosynthesis. Moderate activity was observed against P388 leukemia in vivo.

Subsequent to elucidation of the structure of folic acid, a large number of potential antagonists of this essential vitamin have been synthesized as candidate chemotherapeutic agents.[1-4] The majority of these antagonists inhibit dihydrofolate reductase.[5] Since most folate-metabolizing enzymes utilize folate in its tetrahydro form,[6] it has been suggested that further advances in therapeutic specificity might be achieved with folate antagonists targeted to enzymes other than dihydrofolate reductase.[7] As part of a program to develop inhibitors of N^5,N^{10}-methylenetetrahydrofolate dehydrogenase,[8,9] we became interested in preparing analogues of 5,6,7,8-tetrahydrofolic acid (THFA) as candidate inhibitors of this dehydrogenase as well as other enzymes in the folate metabolic pathways.[8] Compound 1 [*N*-[4-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]-L-glutamic acid, 5-DACTHF, 543U76] was envisaged as an attractive candidate THFA antagonist because (1) it lacked the tetrahydropyrazine ring that is often a source of chemical instability; (2) it retained the molecular elements thought to be important for enzyme binding;[10] and (3) it lacked the 1,2-diaminoethane moiety necessary for substrate activity. Pyrimidine analogues of THFA with antimicrobial activity have been reported by Tong et al.[11,12] Baker's group prepared analogues similar to 1 in which the 4-amino group was replaced with methyl or phenyl.[13,14] These latter compounds had both dihydrofolate reductase and N^5,N^{10}-methylenetetrahydrofolate dehydrogenase inhibitory activity. We now report the synthesis of 5-DACTHF (1) and several derivatives. Data on their (1) activity against folate-utilizing enzymes, (2) cell culture cytotoxicity, and (3) evaluation against leukemia P388 are presented.

Chemistry

The pyrimidinylpropionaldehyde acetal 5 was prepared in two steps from ethyl cyanoacetate and 3-chloropropionaldehyde diethyl acetal via the cyanoester 4 (Scheme I). The amino groups in 5 were protected by acetylation with acetic anhydride to give the triacetyl aminopyrimidine 6. If 6 was heated in water at 100 °C, a water-soluble product was isolated that reacted with aniline in the presence of sodium cyanoborohydride[15] to give the (anilinopropyl)pyrimidinone 9. The absence of an aldehyde proton in the NMR suggested that the water-soluble product was not an aldehyde. Authentic aldehyde 7 was isolated after treatment of 6 with water at ambient temperature. Hot water hydrolysis of 7 gave the same product as was obtained from 6 at 100 °C. Structure 8, an N-acetylated hemiaminal, was assigned to this water-soluble material on the basis of its mass spectrum, proton NMR, and ^{13}C FT NMR spectra. Facile hydrolysis of a similar pyrimidinylpropionaldehyde acetal has been reported.[13]

Reaction of 8 with dimethyl *N*-(4-aminobenzoyl)-L-glutamate[16] gave the desired diacetylated analogue 11. The glycine analogue 13 was obtained similarly from *N*-(4-aminobenzoyl)glycine methyl ester[17] and 8. Selective

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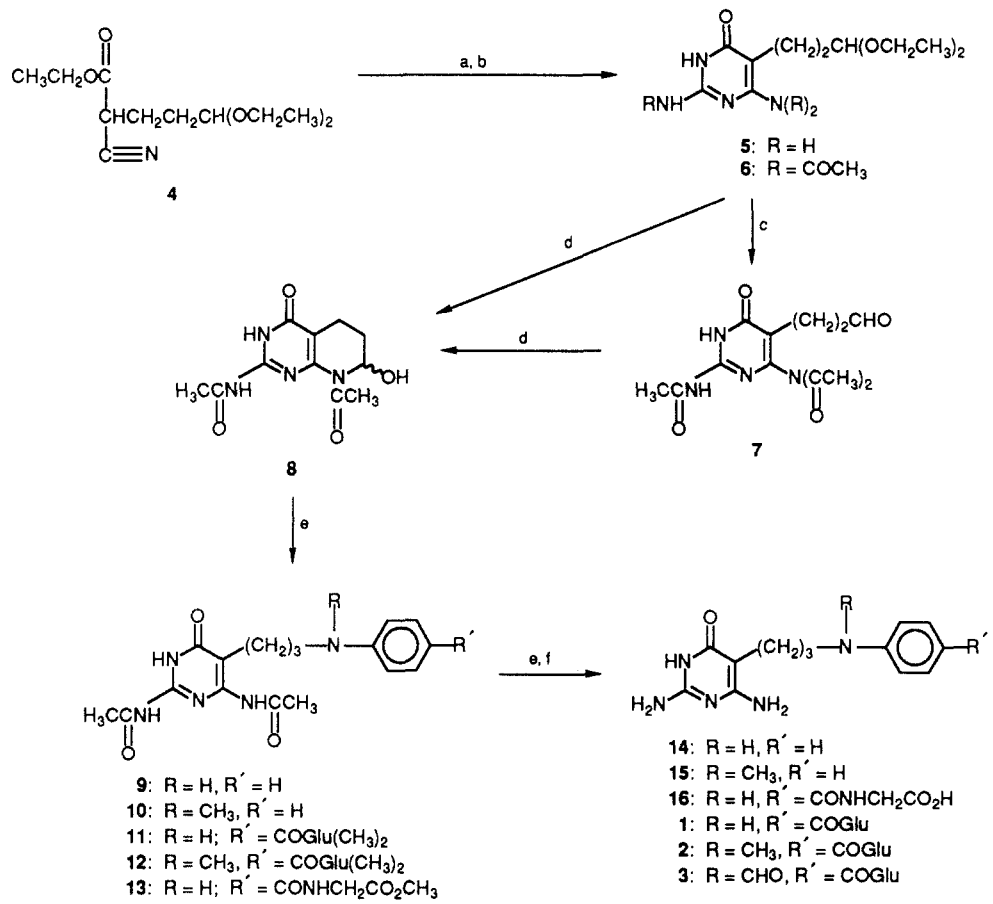
Scheme I[a]



[a] Glu = L-glutamic acid; Glu(CH3)2 = L-glutamic acid dimethyl ester. (a) Guanidine, NaOMe, EtOH; (b) Ac_2O, pyridine; (c) H_2O, 20 °C; (d) H_2O, 100 °C; (e) see Experimental Section; (f) NaOH, 60 °C.

methylation of the anilino nitrogen in **9** was effected by reductive alkylation[15] with aqueous formaldehyde to give **10**. This structure was established unequivocally by the preparation of **10** from **8** and *N*-methylaniline. When this reductive alkylation was extended to **11**, compound **12** was obtained as a homogeneous oil in high yield. Removal of the acetyl groups on **9**, **10**, **11**, **12**, and **13** was readily accomplished with hot 1 N sodium hydroxide to give **1**, **2**, **14**, **15**, and **16**. Compounds **1**, **2**, and **16** were sensitive to light, slowly changing to yellow mixtures if not stored in the dark. The formyl analogue **3** was prepared from **1** with formic–acetic anhydride.[18] The NMR spectrum of **3** showed that this compound was a mixture of syn and anti formanilide isomers in a ratio of 9 to 1.

Biological Results and Discussion

The acyclic THFA analogues **1–3** and compound **16** were tested against 5,10-methylenetetrahydrofolate dehydrogenase from *Escherichia coli*,[9] but none was active at 0.1 mM. These compounds were also tested for in vitro antibacterial activity against a panel of organisms and were not inhibitory at 100 μg/mL.[19] However, when **1–3** were tested against Detroit 98 or L cells in cell culture, potent inhibition of growth was observed (Table I). The parent compound **1** caused 50% inhibition of growth at about 0.1 μM. The *N*-methyl analogue **2** was one-eighteenth and one-third as active, respectively, against these two cell lines. The *N*-formyl analogue **3** was the most toxic, with an IC_{50} of 18 nM against L cells. Retention of the intact glutamic acid moiety was critical for potent cytotoxicity; the glycine analogue **16** was nontoxic at 100 μM. Compound **1** was also active against a panel of human tumor cell lines, with the following IC_{50} values: 43 nM vs A-427 lung carcinoma, 37 nM vs MCF-7 breast adenocarcinoma, 45 nM vs WiDr colon adenocarcinoma, 50 nM vs SW 480 colon adenocarcinoma, 47 nM vs CCRF-CEM T-cell acute lymphob-

Table I. Cell Culture Inhibition by 5-DACTHF (1) and Analogues

compound	IC_{50}, μM[a] Detroit 98	L
1	0.11	0.088
2	2.0	0.28
3	0.21	0.018
16	>100[b]	>100[b]
aminopterin	0.002[c]	0.0023

[a] IC_{50} = concentration giving 50% of control value. [b] No inhibition at 100 μM. [c] From: Szybalski, W.; Szybalska, E. H. *Univ. Mich. Med. Bull.* **1962**, *28*, 277.

Table II. Reversal of L Cell Inhibition by 5-DACTHF (1) and Aminopterin with Thymidine, Hypoxanthine, and Leucovorin

additions to medium[a]	% inhibition by 1 (0.2 μM)	aminopterin (0.01 μM)
(control)[b]	60	72
thymidine (20 μM)	59	68
hypoxanthine (37 μM)	0	79
thymidine + hypoxanthine	0	0
leucovorin (0.2 μM)	6	6

[a] 72-h growth in Eagle's MEM plus 10% horse serum. [b] 1.7×10^6 cells per dish, 5-fold higher than the original inoculum.

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Table III. Effects of 5-DACTHF (1) and 3 on Folate-Utilizing Enzymes

enzyme (source)	% inhibition at concn, μM		folate substrate concn, μM[a]	basic assay ref[a]
	1	3		
dihydrofolate reductase (rat liver)[b]	0 at 100	50 at 36	45	35
thymidylate synthase (calf thymus)[b]	0 at 100	0 at 100	200	36
10-formyltetrahydrofolate synthetase (L cell)[c]	0 at 100	0 at 100	100	32
5,10-methylenetetrahydrofolate dehydrogenase (L cell)[d]	0 at 200	0 at 200	100	9
5,10-methenyltetrahydrofolate cyclohydrolase (L cell)[d]	5 at 200	34 at 200	100	37
serine hydroxymethyltransferase (L cell)[c]	7 at 200	13 at 200	160	38
methionine synthase (L cell)[d]	0 at 200	0 at 200	100	39

[a] Assays were performed essentially as described in the references listed, except the folate substrate concentrations were adjusted as noted. [b] Purified enzyme. [c] L cell crude extract proteins, which precipitated between 30% and 60% saturation of ammonium sulfate, were dissolved and dialyzed against 50 mM potassium maleate (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid, 0.1 M 2-mercaptoethanol, and, in the case of serine hydroxymethyltransferase, 0.1 mM pyridoxal phosphate. [d] Cultured mouse L cells were lysed in 50 mM ammonium sulfate–50 mM Tris, pH 7.5, containing 1 mM dithiothreitol. Cell-free supernatants (20 min, 12000*g*) were dialyzed 4 h against 10 mM Tris-HCl (pH 7.4).

Table IV. Inhibition of Glycinamide Ribonucleotide Transformylase (GAR-TFase) and Aminoimidazole Ribonucleotide Transformylase (AICAR-TFase)

compound	IC_{50}, μM[a]			
	GAR-TFase		AICAR-TFase	
	L cell[b]	hog liver	L cell[b]	MOLT-4
1	3.0	2.6	200	94
1 + Glu_1[c]	0.4	NT	9.4	NT
1 + Glu_2[c]	0.34	NT	1.9	NT
1 + Glu_5[d]	0.08	0.26	0.7	0.7
2	4.3	NT	68	NT
3	18.0	7.6	280	574

[a] IC_{50} values were determined as described under Experimental Section. NT = not tested. [b] See footnote *b*, Table III. [c] The diglutamyl and triglutamyl homologues of 1 were prepared by enzymatic synthesis with purified *E. coli* folylpolyglutamate synthetase[40] followed by purification by reversed-phase HPLC (M. Hanlon and R. Ferone, unpublished data). [d] The hexaglutamyl homologue of 1 was chemically synthesized by V. Styles and J. L. Kelley, manuscript in preparation.

lastic leukemia, and 58 nM vs MOLT-4 T-cell acute lymphoblastic leukemia.

The potent dihydrofolate reductase inhibitor, aminopterin, was somewhat more toxic than 1–3 against D-98 and L cells, with an IC_{50} of 2.3 nM against the L cells (Table I). However, cell culture reversal studies (Table II) and enzyme inhibition tests showed that these acyclic THFA analogues were toxic by a mechanism different from that of aminopterin. Reversal of the toxicity of aminopterin required the addition of both hypoxanthine and thymidine to the cell culture medium. The cytotoxicity of the acyclic THFA analogue 1 was reversed by hypoxanthine alone; thymidine had no effect. Addition of calcium leucovorin at 0.2 μM reversed the cytotoxic effect of both 1 and aminopterin. These data suggest that 1 may interfere with de novo purine biosynthesis at a site requiring a reduced folate cofactor. Compound 1 did not inhibit dihydrofolate reductase from rat liver at 0.1 mM, and 3 was only weakly inhibitory, with an IC_{50} of 36 μM (Table III). Neither 1 nor 3 significantly inhibited six other folate-utilizing enzymes listed in Table III, including those involved in the synthesis of N^{10}-formyltetrahydrofolate, the one-carbon donor for the two transformylases in de novo purine biosynthesis.

The observations on the reversal of cell growth by hypoxanthine alone or by leucovorin, coupled with the findings on the lack of inhibition of the folate interconverting enzymes, suggested that the site of action of 1 was interference with de novo purine biosynthesis by inhibition of GAR-TFase or AICAR-TFase or both. Both enzymes were inhibited by 1 and its analogues (Table IV). GAR-TFase was much more sensitive to 1–3 than was AICAR-TFase. The polyglutamylated homologues of 1 were

Table V. Compounds as Substrates for Hog Liver Folylpoly(γ-glutamate) Synthetase (FPGS)[a]

substrate	app K_m, μM	rel V_{max}, %[b]	rel V_{max}/app K_m
aminopterin	24.4	144	5.9
(6*S*)-H_4PteGlu	4.2	130	31.0
5-DACTHF (1)	6.7	100	14.9

[a] Standard FPGS reaction mixes, as described under Experimental Section, were incubated for 3 h with varying levels of the substrates. A low enzyme concentration was used [0.5 nmol of [^{14}C]glutamate added/(h·mL of reaction mix)] in order to limit the reaction to the production of diglutamate products only. [b] V_{max} relative to a control of 50 μM aminopterin included in each experiment.

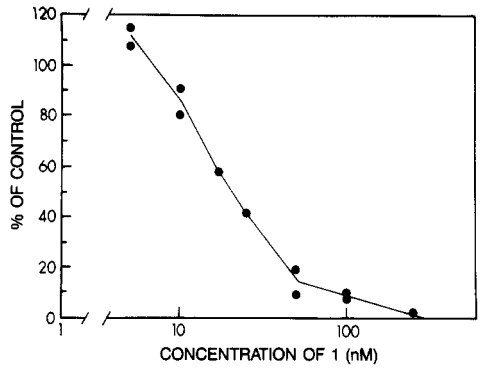

Figure 1. Inhibition of de novo purine biosynthesis by 1. The conversion of [^{14}C]formate to [^{14}C]formylglycinamide ribonucleotide by MOLT-4 cells was monitored as described under Experimental Section.

stronger inhibitors than the parent 1; this increase in potency was more striking with AICAR-TFase than with GAR-TFase. A similar trend was reported by Allegra et al. for methotrexate polyglutamates.[20]

Since polyglutamylation of 1 in vivo would result in activation to more potent enzyme inhibitors and presumably in increased intracellular concentration, it was important to assess the ability of the compound to serve as a substrate for mammalian folylpolyglutamate synthetase (FPGS). Compound 1 was about equal to THFA as a substrate for hog liver FPGS (Table V). Products up to the pentaglutamate were formed in additional experiments with higher FPGS levels and prolonged incubation times (R. Ferone and M. H. Hanlon, unpublished data).

Further evidence supporting the proposed site of action of 1 was obtained in an experiment that determined the

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Table VI. Effect of 5-DACTHF (1) on P388 Tumor Cell Growth in Mice[a]

ip dose, mg/kg	schedule	% increased life span
75	QD, days 1, 5, and 9	0
7.5	b.i.d., days 1–3	+40
5	t.i.d., days 1–4	+22
10	t.i.d., days 1–4	+80

[a] Tested under the protocol of the National Cancer Institute at the Southern Research Institute.[22]

inhibition of the formation of the de novo purine biosynthetic intermediate *N*-formylglycinamide ribonucleotide (FGAR) by cultured MOLT-4 cells. After 24-h growth in the presence of varying concentrations of 1, cells were incubated with [^{14}C]formate and with azaserine, an inhibitor of FGAR metabolism.[21] The formation of [^{14}C]-FGAR was inhibited by 1, with an IC_{50} of 21 nM (Figure 1). This value is close to the concentration needed to inhibit the growth of these cells (IC_{50} of 58 nM, with 72-h continuous drug exposure).

Compounds 1–3 were tested for anticancer activity against leukemia P388 in mice at the Southern Research Institute under the protocol of the National Cancer Institute.[22] Groups of six mice were injected ip with 10^6 leukemia P388 cells on day zero and were treated ip with compounds at various dosing schedules. No significant activity was observed on the day 1, 5, and 9 schedule at doses ranging from 40 to 600 mg/kg, as measured by the increase in survival time of treated animals compared to controls. More frequent dosing of 1 resulted in antitumor activity, with increased life span of 80% by dosing three times a day for 4 consecutive days (Table VI).

Inhibition of de novo purine biosynthesis by folate analogues has been proposed as the site of action of homofolate, its reduced derivatives, and certain one-carbon adducts.[23-26] Cytotoxicity of these compounds was reversed by hypoxanthine or by leucovorin, and they inhibited FGAR accumulation in cultured mammalian cells at micromolar concentrations. Recently, a reduced folate analogue, 5,10-dideazatetrahydrofolate (DDATHF), was shown to inhibit cell growth by this same mechanism at nanomolar concentrations.[27] Both 1 and DDATHF have structural similarities; they are 5-deaza analogues of tetrahydrofolate. DDATHF suppressed the growth of solid tumors,[27] and 1 has significant antitumor activity against P388 leukemia. Further development of these series may reveal whether antifolate inhibitors of de novo purine biosynthesis have clinical usefulness.

Experimental Section

Melting points were determined in capillary tubes on a Mel-Temp block and are uncorrected. NMR data were obtained on a Varian XL-100-15-FT or T-60 spectrometer with TMS as an internal standard. IR spectra were obtained on a Beckman IR8 Spectrophotometer. The ultraviolet absorption spectra were obtained on an Unicam SP 800 spectrophotometer. All analytical samples were assessed for homogeneity by TLC on silica gel or by analysis with a Waters HPLC system on a μBondapak C-18 column with 0.01 M $NaHCO_3$/MeOH. The analytical samples gave combustion values for C, H, and N within 0.4% of theoretical.

Ethyl 2-Cyano-5,5-diethoxypentanoate (4). To a stirred solution of 16.2 g (0.30 mol) of NaOMe in 100 mL of absolute EtOH was added 160 mL (170 g, 1.50 mol) of ethyl cyanoacetate. The solution was spin evaporated in vacuo at 40 °C, and the residual white solid was dissolved in 200 mL of dry DMF. To this solution was added 50 mL (50 g, 0.30 mol) of 3-chloropropionaldehyde diethyl acetal and a crystal of NaI, and the solution was heated on a steam bath with magnetic stirring and protection from moisture for 5 h. The red-brown solution was cooled, poured into 300 mL of ice water, and extracted with Et_2O (6 × 200 mL). The organic phase was washed with H_2O (3 × 50 mL) and brine (50 mL) and then dried ($MgSO_4$). The solution was filtered and spin evaporated in vacuo, and the residue was distilled to give 41.3 g (57%) of a clear liquid, bp 90–108 °C (0.05 mmHg), which was sufficiently pure for the next step. Fractional distillation gave as a main fraction a clear liquid: bp 103–108 °C (0.025 mmHg); NMR ($CDCl_3$) δ 1.20 [t, 6 H, $CH(OCH_2CH_3)_2$], 1.31 (t, 3 H, $CO_2CH_2CH_3$), 1.90 (m, 2 H, CH_2CHCN), 3.60 [m, 5 H, $CH(OCH_2CH_3)_2$ + CHCN], 4.24 (q, 2 H, CO_2CH_2), 4.51 [t, 1 H, $CH(OCH_2CH_3)_2$]; IR (film) 2265, 1750, 1450 cm^{-1}.

3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propionaldehyde Diethyl Acetal (5). To a solution of 22.0 g (407 mmol) of NaOMe in 400 mL of absolute EtOH were added 18.2 g (191 mmol) of guanidine hydrochloride and 46.0 g (189 mmol) of 4. The mixture was refluxed with stirring for 2.5 h, stirred at ambient temperature overnight, and then neutralized with 15 mL of AcOH. The salts were removed by filtration and washed with 100 mL of EtOH. The combined filtrate and wash were spin evaporated in vacuo to give an off-white solid, which was digested with EtOAc and cooled. The solid was collected and washed with EtOAc. This material, which contained NaOAc, was dissolved in 150 mL of 1 N NaOH and then acidified with stirring to pH 5–6 with 10 mL of AcOH. The resultant precipitate was collected, washed with 50 mL of cold H_2O (product partly soluble in water), and dried: yield 29.07 g (60%); mp 178–181 °C. Recrystallization of a portion from EtOAc/EtOH gave the analytical sample: mp 177–178 °C; TLC (C_6H_6/EtOH, 5:1); NMR (DMSO-d_6) δ 1.11 (t, 6 H, CH_3), 1.56 (m, 2 H, CH_2CH_2CH), 2.19 (t, 2 H, CH_2CH_2CH), 3.2–3.7 (m, 4 H, 2 OCH_2), 4.45 (t, 1 H, CH), 5.62 (s, 2 H, NH_2), 5.98 (s, 2 H, NH_2), 9.84 [s, 1 H, HNC(O)]. Anal. ($C_{11}H_{20}N_4O_3$) C, H, N.

3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propionaldehyde Diethyl Acetal (6). A stirred mixture of 7.20 g (28.1 mmol) of 5, 30 mL of dry pyridine, and 30 mL of freshly distilled Ac_2O was heated on an oil bath at ~90 °C for 6 h and then stirred at ambient temperature overnight. Solution occurred within 15 min as a mixture of diacetyl and triacetyl pyrimidinone formed; extended reaction was required to obtain only 6. The solution was spin evaporated in vacuo to give an oil. EtOAc was added and spin evaporated several times until a solid was obtained. The solid was dispersed in cyclohexane and collected: yield 8.88 g (82%); mp 126–137 °C (one spot on TLC). Recrystallization of a portion from cyclohexane/EtOAc gave the analytical sample: mp 138–139 °C; TLC (C_6H_6/EtOH, 5:1); NMR (DMSO-d_6) δ 1.08 (t, 6 H, $2CH_2CH_3$), 1.63 (m, 2 H, CH_2CH_2CH), 2.13 (s, 3 H, Ac), 2.27 (s, 6 H, 2Ac), ~2.2 (m, 2 H, CH_2CH_2CH, superimposed on the acetyl singlets), 3.2–3.7 (m, 4 H, 2 OCH_2), 4.42 (t, 1 H, CH), 11.87 [br s, 2 H, AcNH and HNC(O)]. Anal. ($C_{17}H_{26}N_4O_6$) C, H, N.

3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propionaldehyde (7). A solution of 1.00 g (2.6 mmol) of 6 and 30 mL of H_2O was stirred at ambient temperature for 18 h. TLC showed the absence of 6 and the presence of 7 and 8 in a ratio of approximately 2 to 1. This solution was extracted with Et_2O (3 × 50 mL), and then with $CHCl_3$ (5 × 50 mL). The $CHCl_3$ extracts were combined, washed with 25 mL of H_2O and 25 mL of brine, dried ($MgSO_4$), and spin evaporated in vacuo. Recrystallization of the residue from $CHCl_3$/cyclohexane gave a white powder: yield 0.200 g (25%); mp 164–168 °C; TLC ($CHCl_3$/MeOH, 19:1); NMR ($CDCl_3$) δ 2.28 (s, 3 H, Ac), 2.33 (s, 6 H, 2Ac), 2.67 (t, 4 H, CH_2CH_2), 9.20 (br s, 1 H, AcNH), 9.74 (s, 1 H, CHO), 12.0 (br s, 1 H, ring NH). Anal. ($C_{13}H_{16}N_4O_5 \cdot {}^1/_4H_2O$) C, H, N.

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2-Acetamido-8-acetyl-5,6,7,8-tetrahydro-7-hydroxypyrido[2,3-*d*]pyrimidin-4(3*H*)-one (8). The triacetylated pyrimidinone **6** (25.0 g, 0.66 mol) was heated with 100 mL of H_2O on a steam bath for 4 h. The solution was filtered hot to remove an insoluble byproduct, and the filtrates were spin evaporated in vacuo to a thick syrup. This syrup was successively dissolved in EtOH and then EtOAc followed by spin evaporation until it gave a solid, which was digested with EtOAc. The solids were collected and dried to give a cream colored solid, which was used without further purification: yield 15.1 g (76%); mp 199–201 °C. Recrystallization of a portion from 2-PrOH gave the analytical sample: mp 202–205 °C; TLC (C_6H_6/EtOH, 10:1); NMR (DMSO-d_6) δ 11.75 (br s, 1 H, NH), 11.30 (br s, 1 H, NH, $W_{1/2}$ = 5 Hz), 6.15 (d, 1 H, J = 5.0 Hz, O*H*), 6.06 (m, 1 H, J = 2.7 Hz when D_2O exchange removed OH, C*H*), 2.43 (s, 3 H, CH_3), 2.17 (s, 3 H, CH_3), 2.40–2.10 (m, 2 H, pyrimidine-CH_2), 2.02–1.42 (m, 2 H, CH_2CH_2CH); ^{13}C FT NMR (DMSO-d_6) δ 15.7, 23.9, 27.2, 72.8, 102.2, 147.2, 152.4, 160.4, 171.0, 173.4; MS *m/e* 266, 248. Anal. ($C_{11}H_{14}N_4O_4$) C, H; N: calcd, 21.04; found, 20.35.

2,6-Bis(acetylamino)-5-(3-anilinopropyl)-4(3*H*)-pyrimidinone (9). A mixture of 1.00 g (3.5 mmol) of **8**, 0.51 g (5.5 mmol) of aniline, 1 g of 3-Å molecular sieves, and 50 mL of MeOH was stirred at ambient temperature for 30 min. A crystal of *p*-toluenesulfonic acid and 0.50 g (5.5 mmol) of aniline were added, and the mixture was stirred overnight. One hundred milligrams of $NaBH_3CN$ and 2 mL of AcOH were added, and the mixture was stirred for an additional 15 h. The reaction was diluted with 300 mL of CH_2Cl_2 and filtered through Celite to remove the insolubles. This solution was washed with two 50-mL portions of 5% aqueous $NaHCO_3$ and one 50-mL portion of brine, dried ($MgSO_4$), and spin evaporated in vacuo. The residual syrup was diluted with EtOAc to give a solid, which was again evaporated. The white solids were collected and washed with Et_2O: yield 0.80 g (66%); mp 228–234 °C. Recrystallization from EtOH gave analytically pure material: yield 0.55 g (46%); mp 246–248 °C; TLC (C_6H_6/EtOH, 5:1); NMR (DMSO-d_6) δ 11.50 (br s, 2 H, 2 AcN*H*), 9.64 (br s, 1 H, ring NH), 7.07 and 6.54 (two m, 5 H, ArH), 2.95 (t, 2 H, CCH_2N), 2.43 (t, 2 H, pyrimidine-CH_2), 2.13 and 2.02 (two s, 6 H, 2 Ac), 1.67 (m, 2 H, CCH_2C). Anal. ($C_{17}H_{21}N_5O_3$) C, H, N.

2,6-Bis(acetylamino)-5-[3-(*N*-methylanilino)propyl]-4-(3*H*)-pyrimidinone (10). Method A. A solution of 1.00 g (3.5 mmol) of **8** and 0.375 g (3.5 mmol) of *N*-methylaniline in 25 mL of MeOH and 25 mL of 2-MeOEtOH was stirred with 3 g of 3-Å molecular sieves, 1 mL of AcOH, and 100 mg of $NaBH_3CN$. After 18 h an additional 1 mL of AcOH and 50 mg of $NaBH_3CN$ were added and stirring was continued for 2 h. The mixture was diluted with 500 mL of $CHCl_3$ and filtered to remove the solids. The filtrates were washed with 25 mL of 5% $NaHCO_3$, 25 mL of H_2O, and 25 mL of brine, dried ($MgSO_4$), and spin evaporated in vacuo. Recrystallization from MeOH gave white crystals: yield 0.650 g (52%); mp 210–211 °C; TLC (C_6H_6/EtOH, 5:1); NMR (DMSO-d_6) δ 1.62 (br q, 2 H, $CH_2CH_2CH_2$), 2.02 (s, 3 H, Ac), 2.13 (s, 3 H, Ac), 2.13 (m, 2 H, $ArCH_2$), 2.83 (s, 3 H, CH_3), 3.27 (br s, 2 H, CH_2N), 6.90 (m, 5 H, ArH), 9.67 (br s, 1 H, ring N*H*), 11.6 (br s, 2 H, 2 N*H*Ar). Anal. ($C_{18}H_{23}N_5O_3$) C, H, N.

Method B. To a stirred solution of 0.055 g (0.16 mmol) of **9** in 40 mL of CH_3CN was added 0.04 mL (0.5 mmol) of 37% aqueous formaldehyde. After 5 min 0.012 g of $NaBH_3CN$ and one drop of AcOH were added, and the reaction was stirred for 1 h. After dilution with 350 mL of $CHCl_3$, the solution was washed with H_2O and brine, dried ($MgSO_4$), and spin evaporated in vacuo. Recrystallization from Me_2CO gave 0.034 g (60%) of **10**, which was identical with that prepared by method A according to TLC and mixed melting point.

Dimethyl *N*-[4-[[3-(2,4-Bis(acetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]-L-glutamate (11). A mixture of 2.30 g (7.6 mmol) of **8**, 2.40 g (8.15 mmol) of dimethyl *N*-(4-aminobenzoyl)-L-glutamate,[16] 5 g of 3-Å molecular sieves, 150 mL of MeOH, and 4 mL of AcOH was stirred with protection from moisture for 3 h when "imine" formation was complete. Sodium cyanoborohydride (0.38 g) and 8 mL of AcOH were added to the reaction. After 15 h a trace of intermediate "imine" was detected by TLC and an additional 50 mg of $NaBH_3CN$ was added. After 1 h the reaction was diluted with 500 mL of $CHCl_3$ and filtered through a Celite pad to remove the insolubles. The $CHCl_3$ solution was washed with four 50-mL portions of 5% aqueous $NaHCO_3$, two 50-mL portions of H_2O, and one 50-mL portion of brine, dried ($MgSO_4$), and spin evaporated in vacuo. The resultant foam was triturated with 50 mL of EtOAc to give white crystals: yield 2.75 g (65%); mp 144–150 °C. Recrystallization from EtOH gave chromatographically pure material: yield 1.70 g (40%); mp 135–142 °C. An analytically pure sample was obtained from a previous run: mp 140–143 °C; TLC (C_6H_6/EtOH, 10:1); NMR (DMSO-d_6) δ 11.69 (br s, 2 H, 2 AcNH), 9.68 (br s, 1 H, ring NH), 8.24 (br d, 1 H, ArCON*H*), 7.65 (d, 2 H, ArH), 6.53 (d, 2 H, ArH), 6.17 (br t, 1 H, CH_2NH), 4.39 (m, 1 H, C*H*CO_2CH_3), 3.63 (s, 3 H, CO_2CH_3), 3.58 (s, 3 H, CO_2CH_3), 3.01 (m, 2 H, CH_2NHAr), 2.43 (m, 2 H, C$H_2CO_2CH_3$), ~2.1 (4 H, pyrimidine-CH_2 and CHCH_2, masked by AcNH peaks), 2.14 (s, 3 H, Ac), 2.01 (s, 3 H, Ac), 1.72 (m, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{25}H_{32}N_6O_8 \cdot 0.5H_2O$) C, H, N.

2,6-Diamino-5-(3-anilinopropyl)-4(3*H*)-pyrimidinone (14). A stirred solution of 1.82 g (5.3 mmol) of **9** and 75 mL of 1 N NaOH was heated at 60 °C for 6 h and then stirred at ambient temperature for 18 h. The reaction was neutralized with AcOH, and the precipitated product was crystallized by heating the mixture to 95 °C followed by slow cooling. The tan product was collected [0.60 g (46%)] and twice recrystallizated from EtOAc/Et_2O to give analytically pure material: mp 110–112 °C; TLC (C_6H_6/EtOH, 5:1); NMR (DMSO-d_6) δ 1.57 (q, 2 H, $CH_2CH_2CH_2$), 2.27 (t, 2 H, $ArCH_2$), 2.93 (q, 2 H, CH_2NH), 5.52 (br s, 1 H, NH), 5.70 (s, 2 H, NH_2), 6.00 (s, 2 H, NH_2), 6.85 (m, 5 H, ArH), 9.93 (br s, 1 H, ring NH). Anal. ($C_{13}H_{17}N_5O \cdot 0.5H_2O$) C, H, N.

2,6-Diamino-5-[3-(*N*-methylanilino)propyl]-4(3*H*)-pyrimidinone (15). A solution of 2.30 g (6.45 mmol) of **10** was dissolved in 100 mL of 1 N NaOH and heated at 60–70 °C for 3 h. The solution was spin evaporated in vacuo to 40 mL and neutralized with 7 mL of AcOH. The solids were collected and recrystallized successively from H_2O and then Me_2CO: yield 1.60 g (91%); mp 175–176 °C. An additional recrystallization gave the analytical sample with unchanged melting point, which contained 0.05 molar equivalent of Me_2CO: TLC (C_6H_6/EtOH, 5:1), NMR (DMSO-d_6) δ 1.50 (br m, 2 H, $CH_2CH_2CH_2$), 2.06 (s, 0.3 H, Me_2CO), 2.83 (s, 3 H, CH_3), 2.17 (t, 2 H, $ArCH_2$), 3.30 (t, 2 H, CH_2N), 5.77 (br s, 2 H, NH_2), 6.00 (br s, 2 H, NH_2), 6.97 (br m, 5 H, ArH), 9.93 (br s, 1 H, ring NH). Anal. ($C_{14}H_{19}N_5O \cdot 0.5Me_2CO$) C, H, N.

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]glycine (16).** A mixture of 2.0 g (7.5 mmol) of **8**, 2.0 g (9.6 mmol) of *N*-(4-aminobenzoyl)glycine methyl ester,[17] 10 g of 3-Å molecular sieves, 50 mL of dry MeOH, 50 mL of 2-MeOEtOH, and 5 mL of AcOH were stirred at ambient temperature for 18 h when "imine" formation was complete. The reaction was diluted with 80 mL of DMSO. Five milliliters of AcOH and 0.30 g (47.7 mmol) of $NaBH_3CN$ were added, and the mixture was stirred for 24 h. The reaction mixture was filtered, and the solids were washed with DMSO. The combined filtrate and wash were spin evaporated in vacuo to give **13** as a tan oil, which was essentially a single spot by TLC. This material was dissolved in 100 mL of 1 N NaOH and heated at 50–60 °C for 18 h. The solution was cooled, stirred for 15 h at ambient temperature, and filtered to remove some insoluble particles. The solution was carefully acidified to pH 3 with dichloroacetic acid/water (1:1) to give a precipitate that was collected and washed with H_2O, EtOH, and finally Et_2O. The product was sucked dry and then dried in vacuo at water reflux with protection from light: yield 0.82 g (31%); mp 200 °C dec; TLC (pyridine/BuOH/H_2O, 1:1:1); NMR (DMSO-d_6) δ 1.58 (m, 2 H, $CH_2CH_2CH_2$), 2.26 (t, 2 H, $ArCH_2$), 3.02 (t, 2 H, CH_2CH_2NH), 3.86 (d, 2 H, CH_2CO_2), 5.79 (br s, 2 H, NH_2), 6.00 (br s, 2 H, NH_2), 6.54 (d, 2 H, ArH), 7.63 (d, 2 H, ArH), 8.33 (t, 1 H, CO_2NH), 11.2 (br s, 1 H, ArNH). Anal. ($C_{16}H_{20}N_6O_4 \cdot H_2O$) C, H, N.

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]-L-glutamic Acid (1).** A stirred solution of 1.60 g (2.89 mmol) of **11**, 50 mL of EtOH, and 100 mL of 1 N NaOH was heated at 50–60 °C for 20 h. The cooled reaction was spin evaporated in vacuo to ~50 mL, cooled, and neutralized to pH 5–6 with concentrated HCl. The resultant white precipitate was collected, washed with H_2O and Et_2O, and dried: yield 0.86 g (66%); mp (sinter ~170 °C) 198–202 °C eff. Recrystallization

from MeOH gave the analytical sample: yield, 0.33 g (39%); mp (forms hard foam ~180 °C) ~200 °C eff; TLC (pyridine/BuOH/H_2O, 1:1:1); UV (0.1 N HCl) λ_{max} 227 (ε 15 700), 272 (ε 20 500) nm; (0.1 N NaOH) λ_{max} 275 (ε 23 900), sh 294 (ε 20 200) nm; $[\alpha]^{25}_D$ +6.8° (c 0.99, DMF); NMR (DMSO-d_6) δ 8.07 (d, 1 H, ArCONH), 7.65 (d, 2 H, ArH), 6.54 (d, 2 H, ArH), 6.22 (br s, 1 H, CH_2NH), 5.94 (s, 2 H, NH_2), 5.75 (s, 2 H, NH_2), 4.34 (m, 1 H, $CHCO_2H$), 3.03 (m, 2 H, CH_2NHAr), 2.30 (m, 4 H, pyrimidine-CH_2 and CH_2CO_2H), 2.03 (m, 2 H, $CHCH_2$), 1.58 (m, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{19}H_{24}N_6O_6 \cdot H_2O$) C, H, N.

N-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]methylamino]benzoyl]-L-glutamic Acid (2). To a stirred solution of 1.99 g (3.6 mmol) of 11 in 50 mL of MeCN and 5 mL of DMF was added 2 mL of 37% aqueous formaldehyde followed by 0.50 g (8.0 mmol) of sodium cyanoborohydride and 2 mL of AcOH. After 18 h the mixture was filtered to remove the solids, and the filtrate was spin evaporated in vacuo. The residue was diluted with 100 mL of ice water and then extracted with four 25-mL portions of CH_2Cl_2. The combined extracts were filtered through glass wool and spin evaporated in vacuo to give 2.1 g of **12** as an oil, which was a single spot on TLC. The oil was dissolved in 50 mL of EtOH and 50 mL of 1 N NaOH and stirred at 70 °C for 20 h. The reaction was cooled and spin evaporated in vacuo to a syrup. The syrup was dissolved in 50 mL of H_2O, cooled on ice, and acidified to pH 3–4 with concentrated HCl. The white solids were collected, washed with water, and finally with ether: yield 1.10 g (67%); mp 190–200 °C (one spot on TLC). Recrystallization from EtOH gave the analytical sample: yield 0.54 g (33%); mp (changes to hard foam 166–168 °C) 198–204 °C; TLC (pyridine/BuOH/H_2O, 1:1:1); UV (0.1 N HCl) λ_{max} 227 (ε 14 700), 271 (ε 18 800) nm; (pH 7 buffer) λ_{max} 280 (ε 23 700), 310 (20 600) nm; (0.1 N NaOH) λ_{max} 274 (ε 17 200), 311 (ε 21 400) nm; $[\alpha]^{25}_D$ +6.0° (c 1.0, DMF); NMR (DMSO-d_6) δ 8.16 (d, 1 H, ArCONH), 7.71 (d, 2 H, Ar), 6.66 (d, 2 H, Ar), 5.94 (s, 2 H, NH_2), 5.76 (s, 2 H, NH_2), 4.36 (m, 1 H, $CHCO_2H$), 3.34 [m, 2 H, $CH_2N(CH_3)Ar$], 2.93 (s, 3 H, NCH_3). Anal. ($C_{20}H_{26}N_6O_6 \cdot {}^1/_2H_2O$) C, H, N.

N-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]formylamino]benzoyl]-L-glutamic Acid (3). A solution of 1.16 g (2.6 mmol) of 1 in a solution prepared from 25 mL of 97% formic acid and 5 mL of acetic anhydride[18] was heated on an oil bath at reflux for 1 h. The reaction was cooled and spin evaporated in vacuo to give a hard foam, which was repeatedly covered with EtOH and reevaporated. The foam was triturated under EtOH with a magnetic stirring bar overnight to give a fine solid, which was collected, washed with EtOH, and sucked dry: yield 1.22 g (98%); mp 130–160 °C eff. Several recrystallizations from EtOH gave analytically pure material: yield 0.496 g (40%); mp 170–180 °C eff; UV (0.1 N HCl) λ_{max} 268 (ε 29 600) nm; (pH 7 buffer) λ_{max} 270 (ε 23 700) nm; (0.1 N NaOH) λ_{max} sh 250 (ε 20 200), 264 (ε 23 500) nm; $[\alpha]^{25}_D$ 0° (c 1.0, DMF); NMR (DMSO-d_6) δ 8.63 (d, 1 H, ArCONH), 8.53 and 8.37 (two s in 9:1 ratio at 29 °C, coalesce to a singlet at 120 °C, 1 H, HCON), 7.93 (d, 2 H, ArH), 7.56 (s, ArH of one formanilide isomer), 7.44 (d, 2 H, ArH), 5.93 and 5.70 (two s, 4 H, two NH_2), 4.41 (m, 1 H, $CHCO_2H$), 3.78 [m, 2 H, $CH_2N(CHO)Ar$]. Anal. ($C_{20}H_{24}N_6O_7 \cdot H_2O$) C, H, N.

Cell Culture Materials and Methods. Detroit 98 cells were a cloned derivative from American Type Culture Collection CCL 18.1 (human sternal marrow). L cells were a cloned derivative from ATCC CCL (C3H/AN mouse connective tissue). These cultures were grown in a modification[28] of Eagle's medium and incubated in a water-jacketed CO_2 incubator at 36 °C and 100% humidity.

Total cell counts (made with an electronic cell counter) were compared between replicate test and control monolayer cultures. Controls showed at least two population doublings in the test period of 70–76 h. Percentages of control were plotted against molar concentration of test compound. IC_{50} values equal the concentration giving 50% of control values.

Human tumor cell lines were maintained in RPMI 1640 medium containing fetal calf serum and 10 nM calcium leucovorin as the folate source. Drugs were added 2 h after seeding, and growth inhibition was assessed 72 h later.

Enzyme Assays. Hog Liver Folylpoly(γ-glutamate) Synthetase (FPGS). Hog liver FPGS was purified by the method of Cichowicz and Shane[29] up to the fourth step (phosphocellulose column). It was concentrated by precipitation in 60% ammonium sulfate, dialyzed against 0.1 M Tris-HCl (pH 8.4) containing 50 mM 2-mercaptoethanol and 20% glycerol, and stored at −80 °C. The enzyme was assayed by the charcoal absorption method,[30] in modified reaction mixtures with KCl reduced to 20 mM and 50 μM aminopterin as the standard substrate.

Hog Liver Glycinamide Ribonucleotide Transformylase (GAR-TFase). A streptomycin sulfate treated hog liver homogenate prepared as above for FPGS was fractionated for FPGS (0–40%) and GAR-TFase (40–60%) activity. The 40–60% ammonium sulfate fraction was dialyzed against 37.5 mM potassium phosphate buffer, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol (buffer A), and GAR-TFase was purified on a 10-formyl-5,8-dideazafolate–Sepharose column as described.[31] It was assayed by a modification of the spectrophotometric assay of Smith et al.[32] The assay contained 75 mM Hepes–NaOH, pH 8.5, 50 mM 2-mercaptoethanol, 250 μM α,β-GAR, 0.5 mg/mL bovine serum albumin, 20 μM (6R)-10-CHO-H_4-folate, and enzyme; the reaction was begun with enzyme in a total volume of 400 μL and incubated at 30 °C in 0.5-cm path length cuvettes ($\Delta\epsilon_{295}$ = 18 900 M^{-1} cm^{-1}). The concentration of inhibitor necessary to reduce the rate by 50% (IC_{50}) was estimated from plots of the percentage inhibition vs the logarithm of inhibitor concentration.

MOLT-4 Aminoimidazole Ribonucleotide Transformylase (AICAR-TFase). The enzyme was partially purified from MOLT-4 cells by a modification of the procedure of Mueller and Benkovic.[33] Harvested, washed cells (2.8×10^9) were disrupted by sonication in 12 mL of buffer A plus 25 μg/mL α-1-antitrypsin, 20 μg/mL aprotinin, 13.3 μg/mL pepstatin A, and 1.73 mg/mL benzamidine and were centrifuged for 30 min at 40 000 rpm. The resultant supernatant was passed through a 10-formyl-5,8-dideazafolate–Sepharose column, as described above, to remove GAR-TFase. The column effluent and an 8-mL wash in buffer A were combined and further purified for AICAR-TFase by protamine sulfate and by hydroxyapatite column chromatography.[33] The active fractions from the column were pooled, concentrated by ultrafiltration (American YM10 membrane), and stored at −80 °C with 1.67 mg/mL bovine serum albumin. The continuous spectrophotometric assay was utilized,[34] with 400-μL reaction mixtures containing 100 mM Tris-HCl, pH 8.5, 25 mM KCl, 0.3 mg/mL bovine serum albumin, 50 μM AICAR, 0.5 mM dithiothreitol, 75 μM (6R,S)-10-CHO-THFA, enzyme, and varying concentrations of the compound tested in 0.5-cm path length cuvettes at 30 °C.

N-Formylglycinamide Ribonucleotide (FGAR) Accumulation in Cultured MOLT-4. Purine de novo biosynthesis was

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assessed in MOLT-4 cells by culture by determining the incorporation of [^{14}C]formate into [^{14}C]FGAR. After 24-h incubation in the presence of varying concentrations of 1, cells were resuspended in fresh medium [RPMI 1640 (minus folate) plus 10 nM calcium leucovorin, 10% fetal calf serum, and 0.05 mg of gentamycin/mL], and 0.1 mM azaserine was added. After 15-min incubation [^{14}C]formate was added (0.25 μCi/mL; 1 mM final concentration), the cells were incubated an additional 60 min, and [^{14}C]FGAR was determined.[21]

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Antitubulin Effects of Derivatives of 3-Demethylthiocolchicine, Methylthio Ethers of Natural Colchicinoids, and Thioketones Derived from Thiocolchicine. Comparison with Colchicinoids

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Esterification of the phenolic group in 3-demethylthiocolchicine and exchange of the *N*-acetyl group with other *N*-acyl groups or a *N*-carbalkoxy group afforded many compounds which showed superior activity over the parent drug as inhibitors of tubulin polymerization and of the growth of L1210 murine leukemia cells in culture. A comparison of naturally occuring *Colchicum* alkaloids with thio isosters, obtained by replacing the OMe group at C(10) with a SCH_3 group, showed the thio ethers to be invariably more potent in these assays. The comparison included 3-demethylthiodemecolcine prepared from 3-demethylthiocolchicine by partial synthesis. Thiation of thiocolchicine with Lawesson's reagent afforded novel thiotropolones which exhibited high antitubulin activity. Their structures are fully secured by spectral data. Colchicine and several of its analogues show good antitumor effect in mice infected with P388 lymphocytic leukemia, and all of them show high affinity for tubulin and inhibit tubulin polymerization at low concentration. Consequently, antitubulin assays with this class of compounds can serve as valuable prescreens for the initial evaluation of potential antitumor drugs.

Colchicine (1), a major alkaloid from *Colchicum autumnale*, has antitumor properties but is too toxic to be of value as a clinical antineoplastic agent.[1] The synthesis of analogues obtained by modifying the structure of colchicine has afforded many compounds with improved properties,[2-5] including 3-demethylthiocolchicine (7), which showed broad-spectrum antitumor activity in in vivo systems.[4,5] We decided to further explore the lead provided by 7 and to prepare ester analogues and modified amides of 7. Thiocolchicine (6), studied extensively by Velluz and Muller in France in the early 1950s,[6-9] is a slightly less active antitumor agent than colchicine (1),[4] whereas its 3-demethyl congener 7 has superior antitumor activity.[4,5] Further evaluation of methylthio ether analogues of natural congeners of colchicine (colchicinoids) to assess the significance of this substitution therefore seemed warranted. For this purpose several methylthio ethers prepared earlier were reevaluated. In addition, thiocornigerine (8),[10] prepared here from cornigerine (2),[1] and 3-demethylthiodemecolcine (9), synthesized from thiocolchicine (6), were included in the comparison. Compound 9 is a thioether analogue of 3-demethyldeme-

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