Design and Synthesis of Histidine Analogues of Folic Acid and Methotrexate as Potential Folylpolyglutamate Synthetase Inhibitors

Zhenmin Mao, Jianping Pan, and Thomas I. Kalman*

Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14260

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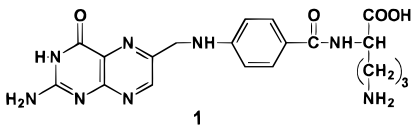
Folylpolyglutamate synthetase (FPGS) is reponsible for the conversion of naturally occurring folates and antifolates to their poly-γ-glutamyl derivatives, which are the forms required for intracellular retention of folates and are also the preferred substrates (cofactors) for most folate-dependent enzymes. Folate and methotrexate analogues **6** and **4**, with L-histidine in place of L-glutamate, were designed and synthesized as potential FPGS inhibitors. Target compound **5**, the N^{τ}-(carboxymethyl)-L-histidine derivative of **4**, was also prepared. Compounds **4** and **5** inhibited the growth of L1210 cells (IC_{50} values: 0.091 and 0.15 μM, respectively) and were potent inhibitors of L1210 dihydrofolate reductase. No significant inhibition of FPGS by **4**, **5**, or **6** was observed at the high pH of the standard enzyme assay. This could be the consequence of a lack of protonation of the basic side chains, which is likely to be required for FPGS inhibitory activity. The observed cytotoxicity indicates that partial protonation of the imidazole ring permits cellular uptake of the analogues.

Polyglutamylation is an essential process for cellular retention and efficient utilization of folates and antifolates. Most folate-requiring enzymes prefer the poly-γ-glutamate forms of folates as cofactors with a chain of four or more glutamate residues.[1−3] The enzyme responsible for the polyglutamylation process is folylpoly-γ-glutamate synthetase (FPGS),[4−7] which requires L-glutamic acid, ATP, and Mg^{2+} to carry out chain elongation.[8] The importance of this enzyme is clearly illustrated by the auxotrophy of a Chinese hamster ovary cell mutant (AUXB1) which has no measurable activity of FPGS and, as a consequence, requires end products of folate-dependent pathways for survival.[9]

Folate analogues targeting FPGS have been studied widely as potential anticancer agents.[10−16] The most potent FPGS inhibitors are folate analogues in which the glutamic acid moiety is replaced by L-ornithine, *e.g.*, N^{α}-pteroyl-L-ornithine (**1**).[14e] Ornithine derivatives of methotrexate (MTX; see **2**, **3**), aminopterin, and other antifolates have also been synthesized.[14a,c,d] In comparison to their acidic side chain counterparts, compounds with the basic ornithine side chain have greater affinities toward FPGS.[13] It is likely that the protonated δ-amino group of ornithine is responsible for enhanced FPGS inhibitory activity. In contrast, protonation of the basic side chain reduces the cytotoxicity of ornithine derivatives of antifolates, presumably by interference with cellular uptake.[12−16] It was of interest to investigate whether a lowering of the basicity of the amino acid side chain and consequently the positive charge density would improve cellular transport without compromising FPGS inhibitory activity. To that end, analogues of folic acid and methotrexate with L-histidine and N^{τ}-(carboxymethyl)-L-histidine side chains (**4**−**6**) were synthesized and tested for inhibition of FPGS and cell growth *in vitro*.



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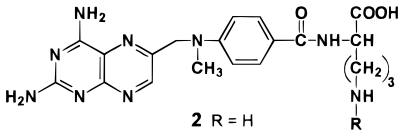

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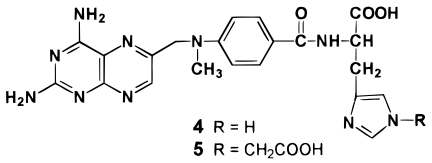
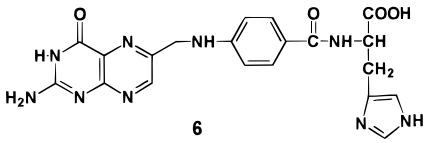
3 R = CH_2COOH



4 R = H
5 R = CH_2COOH



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The pK_a of the imidazole ring of histidine is 5.97,[17a] substantially lower than that of the δ-amino group of ornithine (10.76),[17b] permitting the existence of significant fractions of both unprotonated and protonated species of compounds **4**−**6** at physiological pH. Since ionization may play opposite roles in cellular uptake and enzyme inhibitory activity, both requirements could be simultaneously satisfied. It was postulated that in the enzyme−inhibitor complex the protonated δ-amino group of mAPA-Orn may occupy the binding site of the protonated α-amino group of the incoming glutamate.[12b,c] Since L-histidine may be viewed as a conformationally restricted analogue of L-ornithine, it is possible that its τ-nitrogen can occupy the same FPGS binding site as the δ-amino group of mAPA-Orn (Figure 1). The carboxymethyl group at this position (see **5**) incorporates the α-CH-COOH moiety of the incoming glutamate and was designed to provide additional interactions at the substrate binding site.

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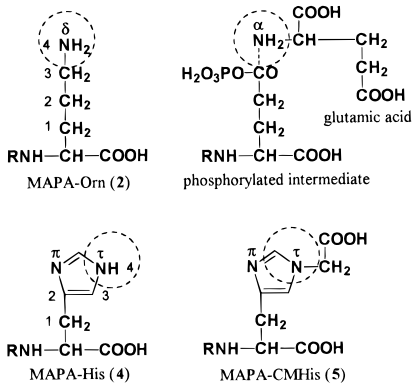
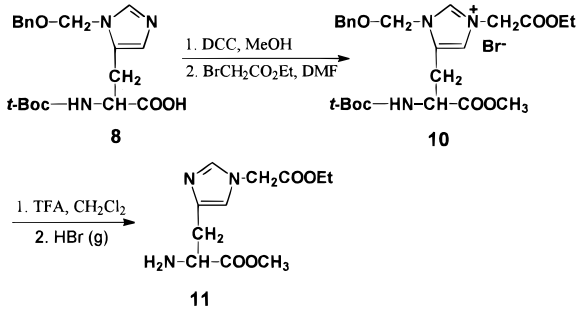


Figure 1. Postulated analogy between the δ- and τ-nitrogens of the ornithine and histidine derivatives of MTX, respectively, and the α-nitrogen of glutamic acid bound to FPGS.

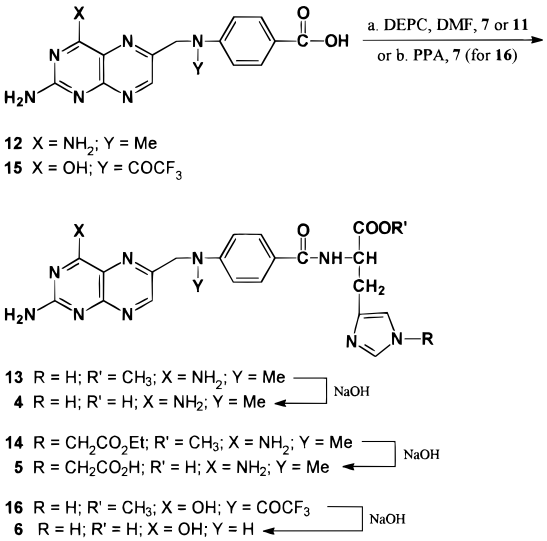
Scheme 1. Synthetic Route to Intermediate **11**



Chemistry

The synthesis of N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-histidine (mAPA-His, **4**) utilized the commercially available L-histidine methyl ester (**7**) as starting material. N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{τ}-(carboxymethyl)-L-histidine (mAPA-CMHis, **5**) was prepared from N^{τ}-[[(ethyloxy)carbonyl]methyl]-L-histidine methyl ester (**11**) as shown in Scheme 1. The synthesis of N^{τ}-[[(ethyloxy)carbonyl)methyl]-L-histidine was reported by Hofmann *et al*. in their studies of *S*-peptide antagonists.[18] In their approach, ethyl bromoacetate was used to alkylate unprotected histidine directly, yielding a mixture of N^{π}-monoalkylated, N^{τ}-monoalkylated, and N^{π},N^{τ}-dialkylated amino acids, necessitating the separation of the two monoalkylated products. In our study, an alternative method was used to obtain exclusively the desired N^{τ}-monoalkylated derivative. N^{π}-(Benzyloxy)methyl (BOM)-protected L-histidine **8** was first esterified in MeOH in the presence of DCC. The resulting methyl ester **9** was alkylated with ethyl bromoacetate in DMF. The yield was satisfactory when DMF was used as the solvent. Other solvents (*e.g.*, THF) gave lower yields and required a longer reaction time. To convert the hygroscopic dialkylated salt **10** to the deprotected intermediate **11**, both the BOM group and the *t*-BOC group of **10** had to be removed. The preferred method of removal of the BOM group is hydrogenation in the presence of a Pd/C catalyst.[19] Other procedures employed trimethylsilyl iodide or bromide,[20] but they may also cleave the ester groups of compound **10**. We found that in trifluoroacetic acid (TFA), anhydrous gaseous hydrogen bromide cleaves the BOM group leaving the ester group intact. Thus, compound **10** was first treated with anhydrous TFA in methylene chloride at 0 °C to remove the *t*-BOC group on the N^{α}-amino group, and then, in the same reaction

Scheme 2. Synthetic Route to Compounds **4**–**6**



mixture, a stream of anhydrous HBr gas was passed for 1 h to remove the BOM protecting group from the N^{π}-position of the imidazole ring to give compound **11** in good yield. The reversed order of treatment (first passage of HBr gas in CH_2Cl_2 followed by TFA) resulted in poor yield.

Pteroic acid and 4-amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) were obtained by enzymatic hydrolysis of folic acid and MTX, respectively, using carboxypeptidase G_1.[21] The synthesis of the protected 4-amino-4-deoxy-N^{10}-methylpteroic acid derivatives **13** and **14** is outlined in Scheme 2. The coupling of mAPA with both L-histidine methyl ester dihydrochloride (**7**) and N^{τ}-alkylated L-histidine ester **11** were achieved using diethyl cyanophosphonate (DEPC) yielding **13** and **14,** respectively. The DEPC coupling reaction gave moderate yields under a variety of conditions. Compounds **13** and **14** were hydrolyzed in aqueous NaOH solution to yield target compounds **4** and **5**.

The synthesis of pteroyl-L-histidine (**6**, Pte-His) was carried out as shown in Scheme 2, starting with N^{10}-trifluoroacetyl-protected pteroic acid **15**, which was prepared by acylation of pteroic acid with trifluoroacetic anhydride. Mixed carboxylic–carbonic anhydride coupling is the method used most often to obtain derivatives of pteroic acid protected at N^{10} by a trifluoroacetyl or formyl group.[12–16] This method involves one or more cycles of addition of *i*-BuOCOCl and the amino acids giving fair to poor yields. The DEPC reagent frequently used in mAPA coupling was previously found by us to cleave the N^{10}-trifluoroacetyl group from the protected pteroate.[21] In the course of investigating different coupling conditions, we found that propylphosphonic anhydride (PPA)[22] is a superior reagent for the coupling of protected pteroic acid and an amino acid ester. The use of this reagent has many advantages over the traditional mixed anhydride method:[12–16] It involves simple reaction conditions and gives better yields and a single reaction product allowing easy workup. Thus, N^{10}-(trifluoroacetyl)pteroic acid was coupled with L-histidine methyl ester (**7**) in the presence of PPA in *N*-methylpyrrolidinone at room temperature to yield the protected compound **16**. Hydrolysis of **16** in aqueous alkaline solution gave the folic acid derivative **6**. Both compounds **4** and **6** were further purified by HPLC on

Table 1. Cell Growth and Enzyme Inhibitory Activity

compound	IC_{50} (μM)[a]		
	L1210 growth	L1210 DHFR[b]	CEM FPGS[c]
mAPA-His (**4**)	0.091 ± 0.020	1.3 ± 0.63	>200
mAPA-CMHis (**5**)	0.15 ± 0.020	0.37 ± 0.096	>200
Pte-His (**6**)	>100	>100	>200
MTX	0.007 ± 0.0015	0.073 ± 0.020	
	1.30[d]		3.2[e]

[a] Concentration required for 50% inhibition. Values are given as mean ± standard deviation (when available). [b] DHFR activity was assayed in permeabilized L1210 cells in the presence of 200 μM H_2folate as substrate.[21] [c] CCRF-CEM FPGS activity was measured at pH 8.5 and 7.9, as described.[14b] [d] From ref 12b. [e] McGuire, J. J. Unpublished results.

a C_{18} silica gel column with a linear water and methanol gradient as the mobile phase. Compound **5** was purified by cation exchange column chromatography (IR-120, H^+-form) using 5% NH_4OH as solvent.

Biological Evaluation and Discussion

Among folate analogues with altered amino acid side chains,[15] those with a basic side chain (*e.g.*, L-ornithine replacing L-glutamate) are the most potent inhibitors of FPGS. However, these analogues suffer from poor cellular uptake which limits their therapeutic utility. We hypothesized that lowering the basicity of the side chain, so that only partial protonation would occur at physiological pH, may eliminate this shortcoming. Accordingly, target compounds **4**−**6** were evaluated as inhibitors of the growth of L1210 murine leukemia cells in culture and of dihydrofolate reductase (DHFR) in permeabilized L1210 cells and as inhibitors of FPGS from CCRF-CEM human leukemia cells. The results are listed in Table 1.

Cell Growth Inhibition Studies. The histidine analogue of MTX (**4**) was 13-fold less active than MTX as an inhibitor of L1210 cell growth but 14-fold more potent than the ornithine analogue of MTX.[12b] The lower growth inhibitory potency of **4** compared to MTX is understandable if one considers that only the unprotonated species of compound **4** may be subject to cellular transport. In contrast, compound **4** should be more active than the fully protonated ornithine analogue **2** which cannot be transported effectively into the cell.

Compound **5** was slightly less active (IC_{50} = 0.15 μM) than its parent compound **4** (0.091 μM). The more polar side chain of **5** may hamper membrane penetration by the analogue resulting in lower cytotoxicity. It would be of interest to directly compare the rate and pH dependence of the cellular uptake of radioactive isotope-labeled analogues, once they become available. Folic acid derivatve **6** did not show significant cell growth inhibition, as expected, since side chain-modified folic acid derivatives generally lack significant cytotoxicity.[16,21] The results suggest that by optimizing the pK_a of potential FPGS inhibitors bearing basic side chains, the opposite ionization requirements of cellular uptake and enzyme inhibitory activity can be satisfied.[23]

Inhibition of DHFR. It is reasonable to assume that the cytotoxicity of side chain-modified MTX analogues is primarily due to inhibition of DHFR, since structural variations at this region have relatively little effect on enzyme inhibitory potency.[15] Indeed, both **4** and **5** showed significant inhibitory activity against DHFR with IC_{50} values of 1.3 and 0.37 μM, respectively. The observation that **5** is more inhibitory to DHFR than **4** but less cytotoxic seems to indicate that **5** is less effectively transported into cells. This conclusion is supported by the finding that in contrast to its higher inhibitory activity in the *permeabilized* system, **5** is 3-fold less active than **4** as an inhibitor in the *intact* cell assay[21,25] (data not shown).

FPGS Inhibition Studies. None of the target compounds showed significant inhibition of FPGS under standard assay conditions (pH 8.5). Since we were interested in the activity of the protonated species, a lowering of the pH of the assay was attempted. However, at the lowest tolerated pH of 7.9, no significant inhibitory activity was observed either. Whereas at pH 7.9−8.5 the δ-amino group of ornithine derivatives is fully protonated (>99%), the imidazole ring of **4**−**6** is not protonated to any significant extent (*ca.* 1%). This may lead to weak binding of the histidine derivatives of FPGS, since protonation of basic side chains appears to be important for FPGS inhibitory activity.[12b]

Experimental Section

Melting points were measured in open-ended capillary tubes on a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Varian (300 MHz) or Varian EM-390 (90 MHz) instrument. IR spectra were taken on a Nicolet 7000 Fourier Transform spectrophotometer. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA. The presence of associated solvent molecules was confirmed by ^{1}H NMR. Thin layer chromatography was performed on Analtech silica gel HLF uniplates. Spots were visualized with the aid of UV light, iodine, ninhydrin, or bromocresol green. TLC systems were used as listed below, unless otherwise stated: (A) 0.1 M KH_2PO_4/H_2HPO_4 (pH 7.4) or (B) 3% NH_4Cl. For silica gel column chromatography, Baker's grade silica gel (200 mesh) was used. HP20 was purchased from Kaser Chemicals, Inc.; DEAE and CC31 cellulose were obtained from Whatman Co. Most of the chemicals used were from Aldrich; PPA (propylphosphonic anhydride) was from Hoechst Chemical, Inc. Triethylamine and *N*-ethylmorpholine were distilled and stored over KOH. All commercial solvents were anhydrous or reagent grades. Carboxypeptidase G_1 was obtained from New England Enzyme Center, Boston, MA. Lyophilizations were carried out on a Virtis Unitrap II freeze-drier. Folate analogues were protected from light whenever possible.

HPLC was performed on a Waters instrument with a semipreparative upgrade. A C_{18} reversed phase silica gel (10 μm) column (7.8 × 30 mm) was purchased from Phenomenex. The mobile phases were (1) 3% CH_3CN and 0.01 M NH_4OAc solution isocratic system and (2) MeOH and H_2O linear gradient system, from H_2O to MeOH. Fractions were monitored by a Rainin Dynemax UV-1 dual-wavelength UV detector at λ = 292 nm. The purity of the target compounds was confirmed by analytical HPLC on a C_{18} reversed phase silica gel column (Phenomenex, 3.9 × 300 mm). The retention time and solvent system used for each compound are reported in their synthesis sections.

***N*$^{\alpha}$-*tert*-Boc-*N*$^{\pi}$-[(benzyloxy)methyl]-L-histidine Methyl Ester (9).** *N*$^{\alpha}$-*tert*-Boc-*N*$^{\pi}$-[(benzyloxy)methyl]-L-histidine (**8**) (500 mg, 1.33 mmol) and 1,3-dicyclohexylcarbodiimide (DCC; 1.0 g, 4.85 mmol) were dissolved in MeOH (30 mL). The reaction mixture was stirred at room temperature for 18 h. The white precipitate of 1,3-dicyclohexylurea formed during the reaction was filtered, and the filtrate was evaporated under reduced pressure. A sticky residue was obtained which was subjected to flash column chromatography on silica gel (CH_2Cl_2:MeOH = 7:1). The pooled fractions were evaporated and dried *in vacuo* to yield a sticky solid (0.39 g, 79%).

To 1.70 g of this solid, 10 mL-hexane and a couple of drops of ether were added. The material was transformed gradually to a white precipitate over a period of hours. After filtration, compound **9** (1.2 g, 70.5%) was obtained as a white powder:

mp 99-101 °C; ^{1}H NMR ($CDCl_3$) δ 7.45 (s, 1H, im-H-2), 7.25 (s, 5H, *Ph*), 6.85 (s, 1H, im-H-5), 5.75 (d, 1H, α-*NH*), 5.25 (s, 2H, Ph-*CH*$_2$), 4.40 (s, 2H, O-*CH*$_2$-N), 4.10 (m, 1H, α-*CH*), 3.75 (s, 3H, COO*CH*$_3$), 3.15 (d, 2H, CH-*CH*$_2$-im), 1.40 (s, 9H, C(*CH*$_3$)$_3$). Anal. ($C_{20}H_{27}N_3O_5$) C, H, N.

***N*$^\alpha$-*tert*-Boc-*N*$^\tau$-[(benzyloxy)methyl]-*N*^{r}-[[(ethyloxy)carbonyl]methyl]-L-histidine Methyl Ester (10).** To a solution of compound **9** (1.0 g, 2.75 mmol) in DMF (30 mL) was added ethyl bromoacetate (3.0 g, 18.0 mmol). The reaction mixture was stirred at 40 °C for 4 h and then at room temperature for another 20 h. The solvent was evaporated, and the residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH from 10:1 to 5:1). The solvent of the pooled fractions was evaporated. After drying the residue *in vacuo*, the hydrobromide salt of compound **10** (1.5 g, 80.5%) was obtained as a hygroscopic white solid: ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 1H, im-H-2), 7.55 (s, 1H, im-H-5), 7.30 (s, 5H, *Ph*), 5.85 (s, 2H, Ph-*CH*$_2$), 5.35 (s, 2H, N-*CH*$_2$-COOEt), 4.71 (s, 2H, O-*CH*$_2$-N), 4.20 (m, 3H, α-*CH* + O*CH*$_2$CH$_3$), 3.75 (s, 3H, COO*CH*$_3$), 3.35 (d, 2H, CH-*CH*$_2$-im), 1.70 (s, H_2O), 1.41 (s, 9H, C(*CH*$_3$)$_3$), 1.32 (t, 3H, OCH$_2$-*CH*$_3$). Anal. ($C_{24}H_{33}N_3O$·HBr·0.5H_2O), C, H, N.

***N*^{r}-[(Ethyloxy)carbonyl]methyl]-L-histidine Methyl Ester (11).** To a solution of compound **10** (1.10 g, 1.98 mmol) in methylene chloride (30 mL) was added trifluoroacetic acid (30 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Anhydrous HBr gas was passed through for 1 h at 0 °C. The reaction mixture was then evaporated to dryness *in vacuo*. The light yellow residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 5:1). After removal of the solvents and drying *in vacuo*, the hydrobromide salt of compound **11** (0.52 g, 63%) was obtained as a white hygroscopic solid: mp 267−269 °C; ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 1H, im-H-2), 7.40 (s, 1H, im-H-5), 5.10 (s, 2H, im-N-*CH*$_2$-COOEt), 4.50 (m, 1H, α-*CH*), 4.25 (m, 2H, O*CH*$_2$CH$_3$), 3.80 (s, 3H, COO*CH*$_3$), 3.30 (d, 2H, CH-*CH*$_2$-im), 1.30 (t, 3H, CH$_2$-*CH*$_3$). Anal. ($C_{11}H_{17}N_3O_4$·2.5HBr) C, H, N.

***N*$^\alpha$-(4-Amino-4-deoxy-*N*^{10}-methylpteroyl)-L-histidine Methyl Ester (13).** 4-Amino-4-deoxy-*N*^{10}-methylpteroic acid (mAPA, **12**; 330 mg, 1 mmol) was suspended in anhydrous DMF (7 mL); Et_3N (0.3 mL, 2.2 mmol) and DEPC (0.15 mL) were added. The reaction mixture was stirred at room temperature under nitrogen for 7 h. To the clear dark red solution were added L-histidine methyl ester hydrochloride salt (**7**) (363 mg, 1.5 mmol) in DMF (5 mL) and Et_3N (0.625 mL, 4.5 mmol). The reaction mixture was kept stirring for 72 h. All solvents were removed *in vacuo*, and the residue was subjected to CC31 cellulose column chromatography (2 × 6 cm). The column was washed with cold 0.1 N NaOH followed by H_2O (100 mL) and eluted with MeOH (150 mL). The combined MeOH fractions were evaporated to dryness. The residue was further purified by flash column chromatography on silica gel ($CHCl_3$:MeOH = 7:1). The pooled fractions were collected, concentrated, and dried *in vacuo* to afford **13** (165 mg, 34.7%) as a yellow solid: mp >230 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.60 (s, 1H, H-7), 7.75 (d, 2H, 2′,6′-H), 7.40 (s, 1H, im-H-2), 6.85 (d, 2H, 3′,5′-H), 6.60 (s, 1H, im-H-5), 4.81 (s, 2H, 9-*CH*$_2$-N), 4.10 (m, 1H, α-*CH*), 3.75 (s, 3H, COO*CH*$_3$), 3.25 (s, 3H, 10-*CH*$_3$), 3.15 (t, 2H, CH-*CH*$_2$-im). Anal. ($C_{22}H_{25}N_{10}O_3$·1.5H_2O) C, H, N.

***N*$^\alpha$-(4-Amino-4-deoxy-*N*^{10}-methylpteroyl)histidine (4).** Compound **13** (50 mg, 0.01 mmol) was suspended in 0.5 N NaOH aqueous solution (1.5 mL). The suspension was stirred at 40 °C under nitrogen for 2 h. The reaction mixture became a clear dark brown solution. This solution was subjected to HPLC on a C_{18} reversed phase silica gel column with a linear gradient of H_2O and MeOH as mobile phase. Pooled pure fractions were freeze-dried after the solvent was removed by rotary evaporation under reduced pressure. Compound **4** (22 mg, 47.8%) was obtained as a yellow solid: TLC (A) R_f = 0.26; mp >280 °C; HPLC (30% aqueous MeOH) retention time, 6.61 min; ^{1}H NMR (DMSO-d_6) δ 8.57 (s, 1H, H-7), 7.88 (s, 1H, im-H-2), 7.66 (d, 2H, 2′,6′-H, J = 8.9 Hz), 6.95 (s, 1H, im-H-5), 6.82 (d, 2H, 3′,5′-H, J = 8.9 Hz), 6.70 (s, 1H, *NH*-CO), 4.78 (s, 2H, 9-*CH*$_2$-N), 4.57 (m, 1H, α-*CH*), 3.37 (broad, H_2O), 3.21 (s, 3H, 10-*CH*$_3$), 3.03 (m, 2H, CH-*CH*$_2$-im). Anal. ($C_{21}H_{22}N_{10}O_3$·3.0H_2O) C, H, N.

***N*$^\alpha$-(4-Amino-4-deoxy-*N*^{10}-methylpteroyl)-*N*^{r}-(carboxymethyl)-L-histidine (5).** To a solution of mAPA (**12**; 1.0 g, 3 mmol) in dry DMF (15 mL) at room temperature under nitrogen were added Et_3N (1.08 mL, 7.7 mmol) and DEPC (0.63 mL, 4.2 mmol). The reaction mixture was stirred for 6 h. Compound **11** (1.3 g, 3 mmol) in DMF (3 mL) and Et_3N (0.6 mL) were added to the dark red solution of activated mAPA. The mixture was stirred at room temperature for another 72 h. MeOH (10 mL) was added, and the mixture was stirred for 30 min. It was evaporated at 40 °C to give a dark gummy residue to which 5% NH_4OH solution (30 mL) was added. A yellow precipitate appeared, which was collected by centrifugation (8000*g*, 30 min, 4 °C). The residue was washed with 0.1 N NaOH (20 mL) and water (20 mL). The solid was dried by lyophilization to give methyl ester **14** (0.63 g, 37.4%) as a brown powder: mp 220 °C dec; ^{1}H NMR (CD_3OD) δ 8.59 (s, 1H, H-7), 7.80 (d, 2H, 2′,6′-H), 7.61 (s, 1H, im-H-2), 6.90 (s, 1H, im-H-5), 6.81 (d, 2H, 3′,5′-H), 4.92 (s, 2H, 9-*CH*$_2$-N), 4.75 (s, 2H, N-*CH*$_2$-COOEt), 4.20 (m, α-*CH* + O*CH*$_2$CH$_3$), 3.75 (s, 3H, COO*CH*$_3$), 3.40 (d, 2H, CH-*CH*$_2$-im), 3.20 (s, 3H, 10-*CH*$_3$), 1.31 (t, 3H, OCH$_2$*CH*$_3$).

Compound **14** (250 mg, 0.44 mmol) was suspended in 1 N NaOH (10 mL). The suspension was stirred at room temperature for about 1 h under nitrogen. The cloudy reaction mixture turned clear and dark red. The solution was filtered to remove any insoluble material; then it was acidified with 2 N HCl to pH 1−2 and filtered. The precipitate was removed by filtration, and the pH of the filtrate was adjusted to 5−6 by adding 0.1 N NaOH. A yellow precipitate appeared which was removed by centrifugation. The solution was applied to an ion exchange column (IR-120, H^+-form), which was washed with water and then with 5% NH_4OH. Fractions containing the product were pooled and concentrated to about 25 mL. The pH was adjusted to 5−6 with 0.1 N HCl. The precipitated yellow solid was allowed to sit at 4 °C overnight and then collected by centrifugation (8000*g*, 30 min, 4 °C). The solid was then washed with water (15 mL), resuspended in water, and lyophilized to yield **5** (152 mg, 66.4%) as a yellow powder: TLC (A) R_f = 0.42; mp >280 °C; HPLC (30% aqueous MeOH) retention time, 6.5 min; ^{1}H NMR (DMSO-d_6) δ 8.61 (s, 1H, H-7), 7.68 (d, 2H, 2′,6′-H, J = 8.9 Hz), 7.65 (s, 1H, im-H-2), 7.03 (s, 1H, im-H-5), 6.82 (d, 2H, 3′,5′-H, J = 8.9 Hz), 6.50 (s, 1H, *NH*-CO), 4.82 (s, 2H, 9-*CH*$_2$-N), 4.81 (s, 2H, N-*CH*$_2$-COOH), 4.55 (m, 1H, α-*CH*), 3.33 (broad, H_2O), 3.22 (s, 3H, 10-*CH*$_3$), 3.02 (t, 2H, CH-*CH*$_2$-im). Anal. ($C_{23}H_{24}N_{10}O_5$·2.5H_2O) C, H; N: calcd, 24.77; found, 24.31.

***N*$^\alpha$-[*N*^{10}-(Trifluoroacetyl)pteroyl]-L-histidine Methyl Ester (16).** *N*^{10}-(Trifluoroacetyl)pteroic acid (**15**; 408 mg, 1 mmol) was dissolved in anhydrous *N*-methylpyrrolidinone (3 mL) and *N*-ethylmorpholine (1.15 g, 10 mmol). L-Histidine methyl ester hydrochloride (**7**) (266 mg, 1.1 mmol) was added, and the reaction mixture was cooled to 0 °C in an ice bath. PPA (1.26 g, 6 mequiv) in ethyl acetate (50%) was added. The reaction mixture was stirred at room temperature under nitrogen for 72 h and cooled in an ice bath; then H_2O (30 mL) was added dropwise. A yellow precipitate formed. The pH was adjusted to 9 with 5% aqueous sodium bicarbonate solution; then the precipitate was filtered and washed with H_2O (10 mL) and ether (15 mL). The solid was dissolved in 0.5 N HCl (5 mL), and the solution was filtered to remove any insoluble material. The filtrate was adjusted to pH 5.5 with 1 N NaOH. The resulting yellow precipitate was collected, washed with cold water (5 mL), and dried *in vacuo* to give **16** (220 mg, 39.4%) as a pale yellow solid: mp 235 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.6 (s, 1H, H-7), 7.6 (d, 2H, 2′,6′-H), 7.4 (s, 1H, im-H-2), 7.0 (d, 2H, 3′,5′-H), 6.65 (s, 1H, im-H-5), 5.05 (s, 2H, 9-*CH*$_2$-N), 4.4 (m, 1H, α-*CH*), 3.75 (s, 3H, COO*CH*$_3$), 3.1 (m, 2H, CH-*CH*$_2$-im).

***N*$^\alpha$-Pteroyl-L-histidine (6).** To compound **16** (90 mg, 0.16 mmol) was added 1 N NaOH (2 mL). The reaction mixture was stirred at 60 °C under nitrogen until most of the starting material disappeared. Some remaining insoluble material was removed by filtration, and the filtrate was adjusted to pH 5.5 by 1 N HCl. The yellow precipitate formed was collected by

centrifugation (7000*g*, 30 min, 4 °C) and washed with H_2O (2 × 5 mL), MeOH (5 mL), and ethyl ether (15 mL) to yield **6** (38 mg, 53.1%) as a yellow solid: TLC (A) $R_f = 0.35$; mp >280 °C; HPLC (H_2O/30% aqueous MeOH gradient) retention time, 18.2 min; ^{1}H NMR (D_2O) δ 8.67 (s, 1H, H-7), 7.71 (s, 1H, im-H-2), 7.64 (d, 2H, J = 9.0 Hz), 6.98 (s, 1H, im-H-5), 6.87 (d, 2H, 3′,5′-H, J = 9.0 Hz), 4.65 (m, 3H, 9-*CH*$_2$-N + α-*CH*), 3.22 (m, 2H, CH-*CH*$_2$-im). Anal. ($C_{20}H_{19}N_9O_4$·1.5H_2O·0.5MeOH) H, N; C: calcd, 50.00; found, 50.48.

Inhibition of Tumor Cell Growth. Duplicate cultures of L1210 murine leukemia cells were exposed to increasing concentrations of test compounds. After 48 h incubation at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, cell numbers were counted with a hemocytometer, and concentrations corresponding to 50% inhibition of control growth (IC_{50} values) were determined. Viability was ≥90% as determined by Trypan blue dye exclusion.

Enzyme Assays. DHFR activity in permeabilized cells was assayed *in situ* in the presence and absence of inhibitors by measuring the ability of dihydrofolate to support the thymidylate synthase-catalyzed release of tritium into water from [5-^{3}H]dUMP[14f] and processed essentially as described.[21,25] FPGS from CCRF-CEM cells was prepared as previously described[24] and assayed[6,24] at pH 8.5, unless stated otherwise.

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