Inhibition of Mammalian Folylpolyglutamate Synthetase and Human Dihydrofolate Reductase by 5,8-Dideaza Analogues of Folic Acid and Aminopterin Bearing a Terminal L-Ornithine

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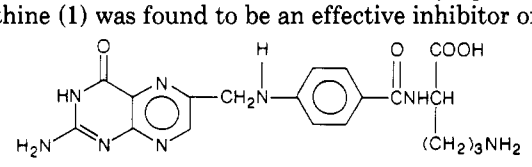
Six new 5,8-dideaza analogues of folic acid and aminopterin containing a terminal L-ornithine residue were prepared by using multistep synthetic sequences. Each was evaluated as an inhibitor of hog liver folylpolyglutamate synthetase and human dihydrofolate reductase. Structural modifications at positions 2, 4, 5, and 10 were included to help define structure–activity relationships for compounds of this type. The compound N^{α}-(4-amino-4-deoxy-5-chloro-5,8-dideazapteroyl)-L-ornithine (**3f**) was identified as the most potent inhibitor of mammalian folylpolyglutamate synthetase reported thus far ($K_i \simeq 2$ nM). Its 4-oxy counterpart, N^{α}-(5-chloro-5,8-dideazapteroyl)-L-ornithine, was only 5-fold less inhibitory than **3f** toward folylpolyglutamate synthetase but was found to be a much weaker inhibitor of dihydrofolate reductase than **3f**.

The enzyme folylpolyglutamate synthetase (FPGS) is responsible for the intracellular conversion of naturally occurring folates to poly-γ-L-glutamyl derivatives. Numerous studies have shown that the formation of these metabolites is essential for the cellular retention of folates in mammalian cells and results in the generation of more efficient substrates for many of the enzymes of one-carbon metabolism.[1-3] The intracellular synthesis of polyglutamates of classical folate antagonists such as methotrexate (MTX) has been shown to be an important determinant of cytotoxicity, since longer chain polyglutamates of MTX are selectively retained by tumor cells in the absence of extracellular drug.[4-7] Recently, impaired glutamylation of MTX has been identified as a primary mechanism of resistance to MTX in a CCRF-CEM human leukemia subline in vitro.[8] Earlier, several mammalian cell lines auxotrophic for the end products of folate metabolism (methionine, glycine, thymidine, and a purine) were shown to be devoid of FPGS activity.[9-11] These results suggest that a potent and selective inhibitor of FPGS could become a useful new chemotherapeutic agent. This paper describes our initial efforts directed toward achieving this objective.

Numerous analogues of folic acid, MTX, and aminopterin modified in the amino acid region have been prepared as potential inhibitors of FPGS. However, all of the potent inhibitors reported thus far having K_i values lower than the K_m values for reduced folate substrates contain a terminal L-ornithine residue. Initially, pteroyl-L-ornithine (**1**) was found to be an effective inhibitor of hog



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liver FPGS (K_i = 5.9 μM), while reduction to its 5,6,7,8-tetrahydro derivative caused a 30-fold reduction in the K_i value.[12,13] Subsequent studies showed that the MTX analogue, in which an L-ornithine replaced the L-glutamate residue, had a K_i value in the same range as **1** against FPGS from K562, CCRF-CEM cells, and rat liver.[14,15] The aminopterin derivative containing an L-ornithine was found to be considerably more potent toward FPGS, having K_i values around 0.2 μM toward both the human and murine liver enzymes.[15,16]

Recently, we reported the substrate activities of a wide variety of 5,8-dideaza analogues of folic acid and aminopterin using homogeneous hog liver FPGS.[17,18] Several of these compounds were highly effective substrates, having activities approaching those of the best reduced folates. The compound 5-chloro-5,8-dideazaaminopterin

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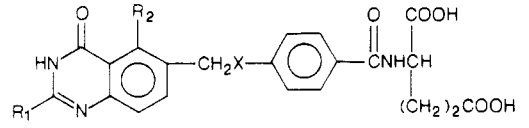
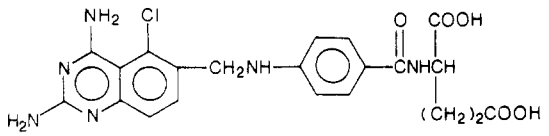
(**2f**) was reported to be the most effective substrate iden-



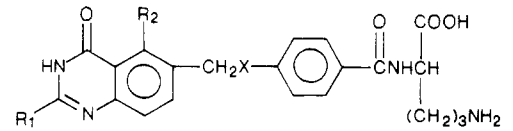
	R_1	R_2	X
2a	H	H	S
b	NH_2	H	S
c	H	H	O
d	NH_2	H	O
e	NH_2	Cl	NH



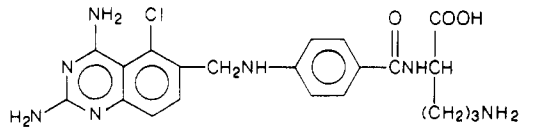
2f

tified thus far. Analogues where S or O replaced N^{10}, **2b** and **-d**, were less efficient than their nitrogen counterparts but were superior to MTX.[17,18] The 2-desamino modifications **2a** and **-c** were recently prepared and found to be 3- to 5-fold more cytotoxic toward L1210 leukemia cells in vitro than their 2-NH_2-containing counterparts **2b** and **-d**.[19] The enhancement in cytotoxicity caused by the removal of the 2-NH_2 group in structurally related compounds was attributed to enhanced cellular uptake.[20]

It was of interest, therefore, to prepare the L-ornithine counterparts of **2a–d** (**3a–d**) for evaluation as inhibitors



	R_1	R_2	X
3a	H	H	S
b	NH_2	H	S
c	H	H	O
d	NH_2	H	O
e	NH_2	Cl	NH

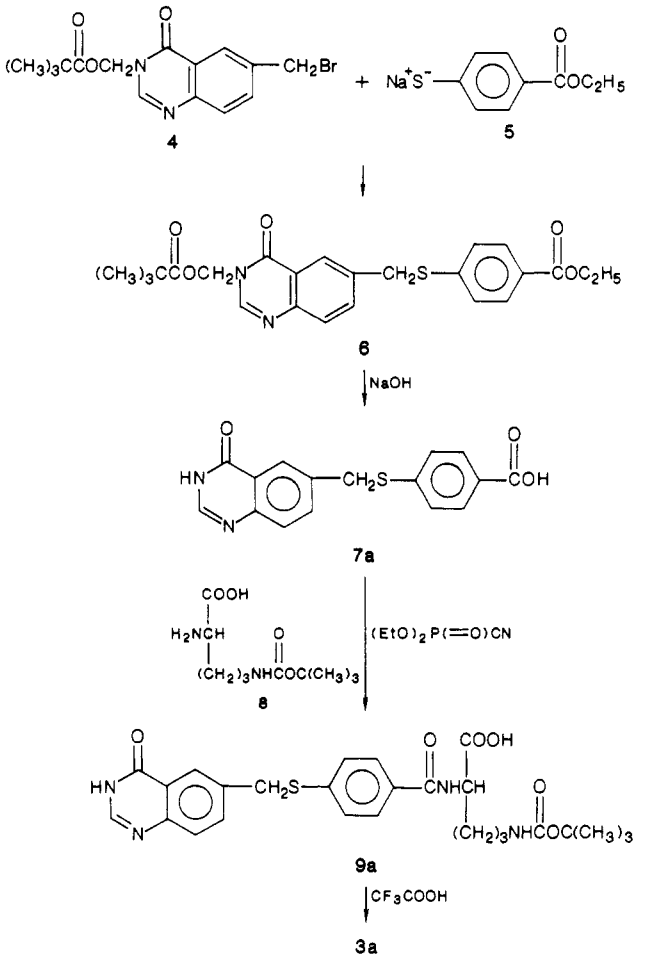


3f

of FPGS. Also synthesized were the 5-chloro folate and aminopterin modifications bearing a terminal L-ornithine, **3e** and **-f**. In previous studies, the presence of a chlorine at position five of the quinazoline nucleus consistently produced the best substrates for FPGS within a given set of analogues.[17,18] During the course of these studies, 5,8-dideazapteroyl-L-ornithine was reported to have a K_i toward human FPGS in the 0.15 μM range.

Chemistry. The preparation of 2-desamino-10-thia-5,8-dideazapteroyl-L-ornithine (**3a**) was facilitated by the recent description of the synthesis of 6-(bromomethyl)-3,4-dihydro-4-oxo-3-[(pivaloyloxy)methyl]quinazoline (**4**).[20] As shown in Scheme I, treatment of **4** with the sodium salt of ethyl *p*-mercaptobenzoate (**5**) gave the fully protected intermediate **6**, which in the presence of base yielded 2-desamino-10-thiapteroic acid (**7a**). Peptide bond formation to N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithine (**8**) using diethyl

Scheme I. Synthesis of N^{α}-(2-Desamino-10-thia-5,8-dideazapteroyl)-L-ornithine



phosphorocyanidate (DEPC) afforded **9a**, which upon treatment with trifluoroacetic acid produced the target compound **3a** in reasonable overall yield. The remaining three new L-ornithine derivatives, **3b–d**, were prepared in an analogous fashion from their corresponding pteroic acid analogues, **7b–d**. The methods employed in preparing these 5,8-dideazapteroates have been described in previous communications.[19,22,23]

The synthesis of the 5-chloro-L-ornithine analogue **3e** was conducted as outlined in Scheme II. The key intermediate 6-[(*p*-carboxyanilino)methyl]-5-chloro-2,4-diaminoquinazoline (**10**) was prepared as described earlier[24] and then subjected to acid-catalyzed hydrolysis to yield 5-chloro-5,8-dideazapteroic acid (**11**), which was converted to the 10-(trifluoroacetyl) derivative **12a** by using anhydrous trifluoroacetic anhydride. The coupling reaction of the latter compound to **8** was conducted by using isobutyl chloroformate as the activating reagent to afford **13a**. This protected intermediate was not purified by silica gel chromatography after it was found that the trifluoroacetyl group was labile under the basic conditions required for elution. Instead, it was treated with ammonium hydroxide to yield **14a**, which was completely characterized. Removal of the *tert*-butyloxycarbonyl group from the N^{δ}-amino

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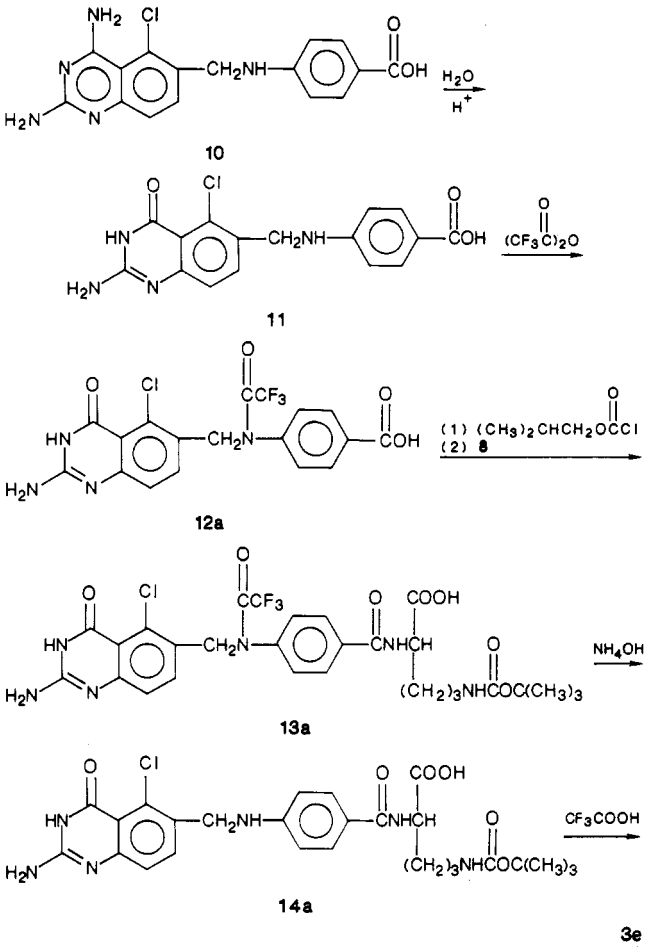
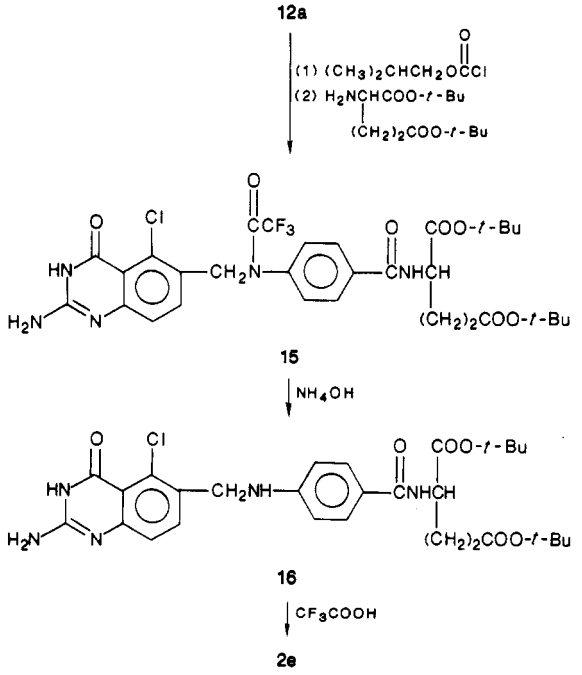
Scheme II. Synthetic Route to N^{α}-(5-Chloro-5,8-dideazapteroyl)-L-ornithine



Scheme III. Synthetic Route to 5-Chloro-5,8-dideazafolic Acid



function of **14a** with trifluoroacetic acid gave the target compound N^{α}-(5-chloro-5,8-dideazapteroyl)-L-ornithine (**3e**). Compound **10** was also converted to its 10-(trifluoroacetyl) derivative 6-[[*p*-carboxy-*N*-(trifluoroacetyl)anilino]methyl]-5-chloro-2,4-diaminoquinazoline (**12b**) by using trifluoroacetic anhydride. Treatment of **12b** in an analogous fashion to that shown in Scheme II then yielded the target compound, N^{α}-(4-amino-4-deoxy-5-chloro-5,8-dideazapteroyl)-L-ornithine (**3f**).

Compound **12a** was also coupled to di-*tert*-butyl-L-glutamate by using isobutyl chloroformate to afford di-*tert*-butyl-5-chloro-N^{10}-(trifluoroacetyl)-5,8-dideazafolate (**15**) as shown in Scheme III. This intermediate was not characterized but was treated with ethanolic ammonium hydroxide to yield di-*tert*-butyl 5-chloro-5,8-dideazafolate (**16**). Removal of the *tert*-butyl groups with trifluoroacetic acid then gave 5-chloro-5,8-dideazafolic acid (**2e**) in modest overall yield. This compound has been prepared earlier in low yield by the reductive condensation of 2-amino-5-chloro-6-cyano-4-hydroxyquinazoline with diethyl (*p*-aminobenzoyl)-L-glutamate followed by basic hydrolysis of the ester functions.[26]

Table I. Comparison of the Kinetic Constants of 5,8-Dideaza Analogues of Folic Acid and Aminopterin with Their L-Ornithine Counterparts for Homogeneous Hog Liver Folylpolyglutamate Synthetase[a]

	L-glutamate			L-ornithine	
compd	K_m, μM	V_{max}[b]	V_{max}/K_m[b]	K_i, μM	$K_i(1)/K_i$
2a	8.1	50	42		
3a				0.42	14
2b	15[c]	33[c]	17[c]		
3b				2.8	2.1
2c	35	95	21		
3c				1.2	4.9
2d	22[c]	25[c]	8.7[c]		
3d				5.9	1.0
2e	0.3	68	1750		
3e				0.0083[d]	710
2f	0.2	57	2210		
3f				0.0017[d]	3470
PteGlu	93[e]	61[e]	5.1[e]		
1				5.9[e]	1.0
(6*S*)-H_4PteGlu	7.7[e]	100[e]	100[e]		

[a] Standard error of the mean, $K_m < \pm 20\%$, $V_{max} < \pm 10\%$. [b] Relative to results for (6*S*)-H_4PteGlu normalized to 100. [c] Reported previously; cf. ref 18. [d] Evaluated by using 0.4 nM FPGS. [e] Reported previously; cf. ref 13.

Table II. Inhibition of Human Dihydrofolate Reductase by 5,8-Dideaza Analogues of Folic Acid and Aminopterin Containing a Terminal L-Ornithine or L-Glutamate

				I_{50}, μM[a]	
compd	R_1	R_2	X	L-glutamate	L-ornithine
2a	H	H	S	0.65[b]	
3a	H	H	S		3.8
2b	NH_2	H	S	0.33[c]	
3b	NH_2	H	S		1.5
2c	H	H	O	0.26[b]	
3c	H	H	O		3.8
2d	NH_2	H	O	0.17[c]	
3d	NH_2	H	O		0.53
2e	NH_2	Cl	NH	0.10	
3e	NH_2	Cl	NH		0.43
2f	NH_2	Cl	NH	0.0038[c]	
3f	NH_2	Cl	NH		0.0030
MTX				0.0043	

[a] Limits of variability <±15%. [b] Reported previously; cf. ref 19. [c] Reported previously; cf. ref 23.

Biological Evaluation. The six new L-ornithine derivatives, **3a–f**, were evaluated as inhibitors of hog liver FPGS, and the K_i values obtained are presented in Table I. The value obtained earlier for **1** is included as a point of reference. Also presented are the kinetic constants for each of the corresponding compounds containing a ter-

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minal L-glutamyl residue. The kinetic parameters for **2a**, **-c**, and **-e** have not been described previously. Analogues **3a–f** were also evaluated as inhibitors of human (WIL2) dihydrofolate reductase, and the I_{50} values obtained are summarized in Table II together with those for the corresponding L-glutamyl modifications. The value obtained with MTX at the time of assay is included for reference purposes.

Discussion

Turning first to the results presented in Table I, it will be seen that for glutamates containing S or O at position 10, removal of the 2-NH_2 group enhances the substrate activity for FPGS as estimated by V_{max}/K_m by approximately 3-fold. Similarly, the K_i's for the 2-desamino-L-ornithine modifications **3a** and **-c** are approximately 5- to 7-fold lower than their 2-NH_2 counterparts **3b** and **-d**. It may also be concluded that sulfur is superior to oxygen at position 10 for both substrate as well as inhibitory activity. For the FPGS reaction, the pseudo-first-order rate constant, V_{max}/K_m, reflects the on rate of the substrate.[13]

The presence of chlorine at position 5 of 5,8-dideaza analogues having a normal folate configuration at positions 9 and 10 affords extraordinarily effective substrates, since **2e** and **-f** are far superior to (6*S*)-tetrahydrofolate [(6*S*)-H_4PteGlu]. The corresponding L-ornithine derivatives containing a chlorine at position 5, **3e** and **-f**, are excellent inhibitors of FPGS, having K_i values that are up to 100-fold lower than any inhibitor of FPGS reported to date.

Various modifications of the analogue substrates cause relatively minor effects on catalytic efficiency, as reflected by V_{max} values, but cause major changes in substrate binding rates, as reflected by V_{max}/K_m and K_m values. An approximately linear relationship was observed between K_m values for various substrates and K_i values for the analogous ornithine inhibitors, and between V_{max}/K_m values and the reciprocal of K_i values. It appears that superior L-glutamate substrates give rise to more potent L-ornithine inhibitors, and increased efficacy primarily reflects an increased binding rate. The potency of the inhibitor **3f** is somewhat higher than would be suggested by the kinetic parameters for the analogous substrate **2f**. However, it should be noted that because of the very low K_m values for analogues **2e** and **-f**, kinetic parameters for these two substrates had to be determined by using very low enzyme concentrations, close to the limit of sensitivity of the assay, to limit the extent of conversion of substrate to product. As a result, the K_m values for **2e** and **-f** may be somewhat lower than indicated in Table I.

In order for an inhibitor of FPGS to be useful in testing the hypothesis that inhibition of this enzyme can selectively kill tumor cells, it is necessary to develop compounds that have low affinity for other folate-requiring enzymes. Therefore, compounds **3a–f** were also evaluated as inhibitors of human DHFR, and the results are compared with those obtained for the corresponding L-glutamates in Table II. For those compounds having a 4-OH configuration, **3a–e**, the replacement of L-glutamate by L-ornithine causes a 3- to 15-fold decrease in inhibitory potency. On the other hand, the 2,4-$(NH_2)_2$ L-ornithine modification **3f** is equipotent with its L-glutamyl counterpart **2f** and MTX. The high affinity for DHFR exhibited by **3f** is consistent with the earlier observation which showed that compound **10**, which is devoid of an amino acid moiety, was equipotent with MTX toward L1210 leukemia DHFR.[25] Compound **3e**, on the other hand, is only a modest inhibitor of DHFR and, therefore, appears to have the highest degree of selectively for FPGS of the inhibitors described thus far.

Experimental Section

Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, or Atlantic Microlab, Inc., Atlanta, GA. All analytical samples gave combustion values for C, H, and N within ±0.4% of the theoretical values unless stated otherwise. Solvation due to H_2O was confirmed by the presence of a broad peak centered at approximately 3.4 ppm in the ^1H NMR spectra, which was transformed into a sharp singlet (DOH) by the addition of D_2O. The presence of CF_3COOH was confirmed by ^{19}F NMR for each compound that contains CF_3COOH in the empirical formula. All intermediates were free of significant impurities on TLC using silica gel media (Kodak 13181). Free L-ornithine derivatives, **3a–f**, as well as **12a–b**, were assayed on Kodak 13254 cellulose (5% NH_4HCO_3). Column chromatographic separations were performed on Kieselgel 60 (70–230 mesh) obtained from E. Merck and Co. The UV spectra were determined on a Cary 219 spectrophotometer in 0.1 N phosphate buffer, pH 7.0. High-resolution ^1H NMR spectra were acquired on a Bruker AM-300 spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC. NMR values for chemical shifts are presented in parts per million downfield from Me_4Si as the internal standard, and the relative peak areas are given to the nearest whole number. Positive (M + 1) and negative ion (M - 1) FAB spectra were obtained on a VG 70SQ analytical spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC, by Dr. Michael Walla. N^5-(*tert*-Butyloxycarbonyl)-L-ornithine (**8**) was purchased from Bachem, Inc., Torrance, CA. Anhydrous DMF was obtained from Aldrich Chemical Co., Milwaukee, WI.

Synthetic methods for the preparation of the glutamates **2a** and **-c** have recently been reported.[19] Hog liver FPGS was purified to homogeneity as described previously.[27] The specific activity of the purified enzyme with (6*S*)-H_4PteGlu as the folate substrate was 123 μmol of $H_4PteGlu_2$ formed/(h·mg of protein) according to the assay conditions described below. Enzyme activity was measured by the incorporation of [^{14}C]glutamate into products by using unlabeled folate or folate analogue as the substrate. Reaction mixtures (0.5 mL) contained 100 mM Tris–50 mM glycine buffer, pH 9.75 (22 °C), folate analogue (various concentrations), L-[^{14}C]glutamate (2 mM, 2.5 mCi/mmol), ATP (1 mM), $MgCl_2$ (10 mM), KCl (20 mM), 2-mercaptoethanol (100 mM), bovine serum albumin (50 μg), and enzyme. The enzyme concentration was adjusted to limit the conversion of substrate to product to less than 5%. The reaction tubes were capped and incubated at 37 °C for 1 h. The pH of the assay mixture was 9.0 at 37 °C. The reaction was stopped by the addition of ice-cold 30 mM 2-mercaptoethanol (1.5 mL) containing 10 mM unlabeled glutamate, and the labeled analogue product was separated from unreacted labeled glutamate by chromatography on small DEAE-cellulose (Whatman DE-52) columns, as described previously.[28] K_m and V_{max} values were determined by an unweighted nonlinear regression method.[29] Kinetic constants for inhibitors were determined by using various concentrations of PteGlu or (6*S*)-H_4PteGlu as the substrate and fixed concentrations of the inhibitor under study. K_i values were the same with either substrate within experimental error.[13]

Homogeneous DHFR was obtained from human WIL2 cells as described earlier.[30] It was assayed spectrophotometrically at 340 nm by using 9 μM dihydrofolate, 30 μM NADPH, and 0.15 M KCl in 0.05 M Tris buffer (pH 7.4); [DHFR] = 0.0086 μM by MTX titration. The final volume was 1 mL, and the assay was performed at 22 °C after a preincubation period of 2 min. MTX was a gift from Dr. Suresh Kerwar, Lederle Laboratories, Pearl River, NY.

6-[[(4-Carbethoxyphenyl)thio]methyl]-3,4-dihydro-4-oxo-3-[(pivaloyloxy)methyl]quinazoline (6). A solution of diethyl 4,4′-dithiobis[benzoate] (1.40 g, 3.86 mmol) in EtOH (80 mL) was reduced with $NaBH_4$ (0.29 g, 7.67 mmol). After being stirred for 15 min, the solution was added dropwise over a period of 15 min

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to a stirred solution of 6-(bromomethyl)-3,4-dihydro-4-oxo-3-[(pivaloyloxy)methyl]quinazoline, (2.05 g, 5.80 mmol) (4)[20] in DMF (25 mL). After the solution was stirred at ambient temperature for 2 h, the solvent was removed at reduced pressure and the residue partitioned between H_2O and $CHCl_3$. The organic layer was separated and dried over $MgSO_4$. Removal of the solvent gave a residue, which was dissolved in C_6H_6 (2.5 mL) and applied to a silica gel column (33 × 1.8 cm) and then eluted with C_6H_6. Fractions homogeneous by TLC were pooled and evaporated to yield a pale yellow oil, which when dried under vacuum at 50 °C for 15 h solidified to give 2.08 g (79%) of a pale yellow solid: mp 107.5–111.5 °C (preliminary softening); TLC R_f 0.30 (C_6H_6–MeOH, 99:1); NMR (Me_2SO-d_6) δ 1.11 [s, 9, $C(CH_3)_3$], 1.29 (t, 3, CH_2CH_3, J = 7.08 Hz), 4.27 (q, 2, CH_2CH_3, J = 7.02 Hz), 4.55 (s, 2, CH_2S), 5.90 (s, 2, $COOCH_2N$), 7.47 (d, 2, 3′, 5′, J_0 = 8.34 Hz), 7.65 (d, 1, H_8, $J_{8,7}$ = 8.34 Hz), 7.83 (d, 2, 2′, 6′, J_0 = 8.28 Hz), 7.91 (app dd, 1, H_7, $J_{7,8}$ = 8.43 Hz), 8.22 (app d, 1, H_5), 8.44 (s, 1, H_2). Anal. ($C_{24}H_{26}N_2O_5S$) C, H, N.

6-[[(4-Carboxyphenyl)thio]methyl]-3,4-dihydro-4-oxoquinazoline (7a). A mixture containing **6** (1.95 g, 4.29 mmol), EtOH (55 mL), H_2O (55 mL), and 1 N NaOH (17 mL) was stirred at ambient temperature for 42 h. The pale yellow colored solution was filtered, and the pH was adjusted to 5.00 with 1 N HCl. The mixture was stirred for 15 min and then cooled. The product was separated by filtration, washed with H_2O (200 mL), and dried under vacuum, first at 60 °C for 5 h, and then at 100 °C over P_2O_5 for 15 h to give 1.19 g (89%) of an off-white solid: mp 287–291 °C dec (preliminary softening); TLC R_f 0.27; NMR (Me_2SO-d_6) δ 4.52 (s, 2, CH_2S), 7.44 (d, 2, 3′, 5′, J_0 = 8.01 Hz), 7.62 (d, 1, H_8, $J_{8,7}$ = 8.37 Hz), 7.82 (d, 2, 2′, 6′, J_0 = 9.21 Hz, partially superimposed upon dd 1, H_7), 8.06 (s, 1, H_2 or H_5), 8.16 (s, 1, H_2 or H_5), 12.26 (s, 1, lactam NH), 12.91 (br s, 1, COOH). Anal. ($C_{16}H_{12}N_2O_3S$) C, H, N.

N^{δ}-(*tert*-Butyloxycarbonyl)-N^{α}-(2-desamino-10-thia-5,8-dideazapteroyl)-L-ornithine (9a). To a stirred solution of **7a** (0.30 g, 0.96 mmol) in DMF (10 mL) was added a solution of Et_3N (0.19 g, 1.88 mmol) in DMF (1 mL) followed by the addition of a solution of diethyl phosphorocyanidate (DEPC) (0.19 g, 1.17 mmol) in DMF (1 mL). The solution was stirred under N_2 for 1.5 h, at which time N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithine (**8**) (0.25 g, 1.08 mmol) was added as a suspension in DMF (5 mL). After the mixture was stirred for 3 h, the reaction was terminated by the addition of $NaHCO_3$ (0.16 g, 1.92 mmol). After removal of the solvent at reduced pressure, the residual yellow-orange oil was dissolved in $CHCl_3$ (3 mL) and applied to a silica gel column (30 × 1.8 cm) packed with $CHCl_3$. The product was eluted with $CHCl_3$–MeOH, 9:1, and $CHCl_3$–MeOH, 85:15. Fractions homogeneous by TLC were pooled and evaporated to yield 0.34 g of crystalline solid. This was then dissolved in 5% aqueous $NaHCO_3$ (10 mL) and the mixture stirred for 30 min and then filtered to remove insoluble material. The pH of the filtrate was adjusted to 3.50 with 0.2 N HCl. The precipitated product was removed by filtration, washed with H_2O, and dried under vacuum at 50 °C for 16 h to give 0.17 g (31%) of yellow solid: mp 163–164 °C dec; TLC R_f 0.27 ($CHCl_3$–MeOH, 7:3); NMR (Me_2SO-d_6) δ 1.32–1.88 [m, 13, CH_2CH_2 and $C(CH_3)_3$], 2.91 (app q, 2, CH_2NHCOO), 4.27 (br s, 1, orn α-CH), 4.51 (s, 2, CH_2S), 6.81 (app t, 1, NHCOO), 7.43 (d, 2, 3′, 5′, J_0 = 8.43 Hz), 7.61 (d, 1, H_8, $J_{8,7}$ = 8.40 Hz), 7.78 (d, 2, 2′, 6′, J_0 = 8.46 Hz), 7.85 (dd, 1, H_7, $J_{7,8}$ = 8.46 Hz, $J_{7,5}$ = 2.13 Hz), 8.06 (s, 1, H_2), 8.16 (d, 1, H_5, $J_{5,7}$ = 1.83 Hz), 8.51 (d, 1, CONH, J = 7.77 Hz), 12.24 (br s, 1, lactam NH), 12.56 (br s, 1, COOH); FAB/MS 525 (M − 1). Anal. ($C_{26}H_{30}N_4O_6S$·1.75H_2O) C, N; H: calcd, 6.04; found, 5.49.

N^{α}-(2-Desamino-10-thia-5,8-dideazapteroyl)-L-ornithine (3a). Compound **9a** (0.10 g, 0.18 mmol) was dissolved in CF_3COOH (3 mL) and the solution stirred for 2 h. The solvent was removed under vacuum and the residual solid triturated three times with Et_2O. After drying, the resulting solid was dissolved in 10% NH_4OH (14 mL) and the yellow-colored solution stirred at ambient temperature for 1 h. The solvent was removed under vacuum, and the residual traces of NH_3 were removed by entrainment with 3 × 30-mL portions of H_2O. The residual solid was triturated with H_2O (15 mL) and the mixture stirred for 2 h. The compound was removed by filtration, washed with H_2O, and dried under vacuum over P_2O_5 at 65 °C for 20 h to yield 0.061 g (74%) of yellow solid: mp 197–198 °C dec; TLC R_f 0.54 (EtOH–NH_4OH, 8:2); UV λ_{max} 228 (ε 30.5 × 10^3), 276 (16.1 × 10^3) nm; NMR (CF_3COOD) δ 1.96–2.44 (two br s, 4, CH_2CH_2), 3.42 (br s, 2, CH_2NH_2), 4.45 (s, 2, CH_2S), 5.04 (br s, 1, orn α-CH), 6.91 (br s, 2), 7.44 (d, 2, 3′, 5′, J_0 = 8.10 Hz), 7.72 (d, 2, 2′, 6′, J_0 = 8.25 Hz), 7.92 (d, 1, H_7 or H_8, $J_{7,8}$ = 8.52 Hz), 8.22 (d, 1, H_7 or H_8, $J_{7,8}$ = 8.25 Hz), 8.52 (s, 1, H_5), 9.31 (s, 1, H_2); FAB/MS 427 (M + 1), 425 (M − 1). Anal. ($C_{21}H_{22}N_4O_4S$·2H_2O) C, N; H: calcd, 5.67; found, 5.14.

N^{δ}-(*tert*-Butyloxycarbonyl)-N^{α}-(10-thia-5,8-dideazapteroyl)-L-ornithine (9b). To a stirred suspension of 10-thia-5,8-dideazapteroic acid (**7b**) (0.30 g, 0.90 mmol)[23] in DMF (50 mL) at 0 °C was added a solution of Et_3N (0.18 g, 1.80 mmol) in DMF (3 mL) followed by the addition of DEPC (0.18 g, 1.10 mmol) in DMF (5 mL). The mixture was stirred at 0 °C under N_2 for 1.2 h by which time a clear pale yellow solution was obtained. To this was added **8** (0.23 g, 0.99 mmol), and the stirring was continued at 0 °C for 3 h and then at ambient temperature for 1 h. The solvent was removed under vacuum and the residue applied to a column of silica gel (30 × 1.8 cm) packed wtih $CHCl_3$. The product was eluted with $CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5. Appropriate fractions were pooled and evaporated to yield 0.38 g of a colorless solid. Next, the compound was dissolved in 10% NH_4OH (10 mL), and the milky white mixture was stirred at ambient temperature for 0.5 h. The solvent was removed under vacuum and the residual NH_3 removed by entrainment with 3 × 40 mL portions of H_2O. The solid was triturated with H_2O (30 mL) and the product removed by filtration, washed with water, and dried under vacuum over P_2O_5 at 100 °C for 12 h to yield 0.29 g (58%) of white solid: mp 200–203 °C dec; TLC R_f 0.22 ($CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5); NMR (Me_2SO-d_6) δ 1.00–1.88 [m, 13, $C(CH_3)_3$ and CH_2CH_2], 2.90 (br s, 2, CH_2NHCOO), 4.24 (br s, 1, orn α-CH), 4.38 (s, 2, CH_2S), 6.52 (br s, 2, 2-NH_2), 6.80 (br s, 1, NHCOO), 7.13 (d, 2, H_7 or H_8, $J_{7,8}$ = 8.25 Hz), 7.41 (d, 2, 3′, 5′, J_0 = 8.07 Hz), 7.58 (d, 1, H_7 or H_8, $J_{7,8}$ = 8.76 Hz), 7.78 (d, 2, 2′, 6′, J_0 = 7.92 Hz), 7.91 (s, 1, H_5), 8.45 (d, 1, CONH, J = 7.53 Hz). Anal. ($C_{26}H_{31}N_5O_6S$·1H_2O) C, H, N.

N^{α}-(10-Thia-5,8-dideazapteroyl)-L-ornithine (3b). A sample of **9b** (0.10 g, 0.18 mmol) was dissolved in CF_3COOH (3 mL) and the solution stirred for 3 h. The solvent was removed under vacuum and the residue triturated three times with Et_2O. After drying, the solid was dissolved in 10% NH_4OH (14 mL) and the mixture stirred at ambient temperature for 1 h. The solvent was removed under vacuum and the residual traces of NH_3 removed by entrainment with 3 × 30-mL portions of H_2O. The residual solid was triturated with H_2O (10 mL) and isolated by filtration. It was then washed with H_2O and dried under vacuum for 15 h at 45 °C to give 0.069 g (78%) of white powder: mp 289–290 °C dec; TLC, no satisfactory system found; UV λ_{max} 230 (ε 27 × 10^3), 277 (9.6 × 10^3) nm; NMR (CF_3COOD) δ 1.98–2.48 (m, 4, CH_2CH_2), 3.40 (br s, 2, CH_2NH_2), 4.34 (s, 2, CH_2S), 5.03 (br s, 1, orn α-CH), 6.90 (br s), 7.42 (br s, 3, 3′, 5′, and H_8), 7.69 (br s, 2, 2′, 6′), 7.97 (br s, 1 H_7), 8.27 (s, 1, H_5); FAB/MS 442 (M + 1), 440 (M − 1). Anal. ($C_{21}H_{23}N_5O_4S$·2.25H_2O) C, N; H: calcd, 5.75, found, 5.18.

N^{δ}-(*tert*-Butyloxycarbonyl)-N^{α}-(2-desamino-10-oxa-5,8-dideazapteroyl)-L-ornithine (9c). To a stirred solution of 6-[(4-carboxyphenoxy)methyl]-4-hydroxyquinazoline (**7c**) (0.25 g, 0.84 mmol)[19] in DMF (35 mL) was added a solution of Et_3N (0.17 g, 1.68 mmol) in DMF (2.5 mL) followed by the addition of a solution of DEPC (0.28 g, 1.72 mmol) in DMF (2.5 mL). The solution was stirred under N_2 for 3 h, at which time **8** (0.22 g, 0.95 mmol) was added as a suspension in DMF (10 mL). After stirring for 15 h, the reaction was terminated by the addition of $NaHCO_3$ (0.14 g, 1.67 mmol). After removal of the solvent under vacuum, the residual yellow oil was dissolved in $CHCl_3$ (2 mL) and applied to a silica gel column (28 × 1.8 cm) packed with $CHCl_3$. The column was eluted with $CHCl_3$–MeOH, 9:1. Fractions homogeneous by TLC were pooled and evaporated to yield 0.33 g of product. This was then dissolved in 5% aqueous $NaHCO_3$ solution (12 mL) and filtered to remove insoluble particles. The pH of filtrate was adjusted to 3.50 with 0.2 N HCl. The precipitated product was removed by filtration, washed with water, and dried under vacuum at 50 °C for 19 h to give 0.13 g (30%) of yellow solid: mp 130–133 °C dec; TLC R_f 0.28 ($CHCl_3$–MeOH, 7:3); NMR (Me_2SO-d_6) δ 1.22–1.90 [m, 13, CH_2CH_2 and $C(CH_3)_3$], 2.92 (app q, 2, CH_2NHCOO), 4.31 (m, 1, orn α-CH), 5.35 (s, 2, CH_2O), 6.81 (app t, 1, NHCOO), 7.12 (d, 2, 3′, 5′, J_0 = 8.85 Hz), 7.70 (d, 1, H_8,

$J_{8,7}$ = 8.34 Hz), 7.87 (d, 2, 2′, 6′, J_0 = 8.64 Hz, superimposed upon dd, 1, H_7), 8.11 (s, 1, H_2), 8.21 (d, 1, H_5, $J_{5,7}$ = 1.71 Hz), 8.42 (d, 1, CONH, J = 7.77 Hz), 12.31 (br s, 1, lactam NH), 12.44–12.72 (br s, 1, COOH). Anal. ($C_{26}H_{30}N_4O_7 \cdot 0.5H_2O$) C, H, N.

N^{α}-(2-Desamino-10-oxa-5,8-dideazapteroyl)-L-ornithine (3c). Compound **9c** (0.05 g, 0.096 mmol) was dissolved in a mixture of $CHCl_3$ (2 mL) and CF_3COOH (1 mL) and the solution stirred for 4.5 h. The reaction mixture was evaporated at reduced pressure and the residue triturated three times with Et_2O. The solid was dried at 45 °C under high vacuum for 4 h to give 0.032 g (62%) of white powder, mp 152–160 °C dec (preliminary softening). The analytical sample was dried in vacuo, over P_2O_5 at 65 °C for 15 h: TLC R_f 0.25; UV λ_{max} 230 (ϵ 30.4 × 10^3), 256 (23.1 × 10^3) nm; NMR (CF_3COOD) δ 2.04–2.44 (m, 4, CH_2CH_2), 3.44 (br s, 2, CH_2NH_2), 5.08 (br s, 1, orn α-CH), 5.45 (s, 2, CH_2O), 6.93 (br s), 7.19 (d, 2, 3′, 5′, J_0 = 8.79 Hz), 7.87 (d, 2, 2′, 6′, J_0 = 8.70 Hz), 8.05 (d, 1, H_7 or H_8, $J_{7,8}$ = 8.64 Hz), 8.29 (d, 1, H_7 or H_8, $J_{7,8}$ = 8.58 Hz), 8.66 (s, 1, H_5), 9.38 (s, 1, H_2); FAB/MS 411 (M + 1); 409 (M − 1). Anal. ($C_{21}H_{22}N_4O_5 \cdot CF_3COOH \cdot H_2O$) C, H, N.

N^{δ}-(*tert*-Butyloxycarbonyl)-N^{α}-(10-oxa-5,8-dideazapteroyl)-L-ornithine (9d). To a stirred suspension of 10-oxa-5,8-dideazapteroic acid (**7d**) (0.21 g, 0.66 mmol)[22] in DMF (35 mL) at 0 °C was added a solution of Et_3N (0.13 g, 1.31 mmol) in DMF (2.5 mL). This was followed by the addition of a solution of DEPC (0.13 g, 0.79 mmol) in DMF (2.5 mL). The mixture was stirred at 0 °C under N_2 for 1.2 h, by which time it had become clear and colorless. To this was added **8** (0.17 g, 0.72 mmol), and the mixture was stirred at 0 °C for 4 h and then for 7 h at ambient temperature. The reaction was terminated by the addition of $NaHCO_3$ (0.11 g, 1.31 mmol). The solvent was removed under reduced pressure and the residue triturated with H_2O. The resulting solid was separated by filtration, washed with water, and dried under vacuum over P_2O_5 at 65 °C for 17 h. There was obtained 0.31 g (84%) of white powder: mp 177–182 °C dec; TLC R_f 0.13 ($CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5); NMR (Me_2SO-d_6) δ 0.94–1.92 (m, 4, CH_2CH_2), 1.36 [s, 9, $C(CH_3)_3$], 2.91 (br s, 2, CH_2NHCOO), 4.26 (br s, 1, orn α-CH), 5.19 (s, 2, CH_2O), 6.62 (br s, 2, 2-NH_2), 6.80 (br s, 1, NHCOO), 7.09 (d, 2, 3′, 5′, J_0 = 7.38 Hz), 7.21 (d, 1, H_8, $J_{8,7}$ = 8.22 Hz), 7.63 (d, 1, H_7, $J_{7,8}$ = 7.08 Hz), 7.85 (d, 2, 2′, 6′, J_0 = 8.01 Hz), 7.96 (s, 1, H_5), 8.28 (app d, 1, CONH). Anal. ($C_{26}H_{31}N_5O_7 \cdot 2.25H_2O$) C, N; H: calcd, 6.32; found, 5.89.

N^{α}-(10-Oxa-5,8-dideazapteroyl)-L-ornithine (3d). A sample of **9d** (0.15 g, 0.27 mmol) was dissolved in CF_3COOH (3 mL) and the solution stirred at ambient temperature for 2 h. The solvent was removed under vacuum and the residue triturated three times with Et_2O. After drying, the solid was dissolved in 10% NH_4OH (21 mL) and the solution was clarified by centrifugation and decantation. The solvent was removed under vacuum and the residual traces of NH_3 removed by entrainment with 3 × 45-mL portions of H_2O. The residue was then treated with Me_2CO, which was then removed under vacuum. The compound was finally triturated with H_2O (15 mL) and isolated by filtration. It was then washed with H_2O and dried under vacuum over P_2O_5 at 65 °C for 17 h to give 0.063 g (47%) of white solid: mp 190.5–197 °C dec; TLC R_f 0.33 (EtOH–NH_4OH, 8:2); UV λ_{max} 230 (ϵ 29.7 × 10^3), 256 (18.7 × 10^3) nm; NMR (CF_3COOD) δ 2.08–2.52 (m, 4, CH_2CH_2), 3.50 (br s, 2, CH_2NH_2), 5.15 (br s, 1, orn α-CH), 5.42 (s, 2, CH_2O), 7.00 (br s), 7.24 (d, 2, 3′, 5′, J_0 = 8.49 Hz), 7.63 (d, 1, H_8, $J_{8,7}$ = 8.22 Hz), 7.92 (d, 2, 2′, 6′, J_0 = 8.25 Hz), 8.12 (d, 1, H_7, $J_{7,8}$ = 8.76 Hz), 8.49 (s, 1, H_5); FAB/MS 426 (M + 1), 424 (M − 1). Anal. ($C_{21}H_{23}N_5O_5 \cdot 0.5CF_3COOH \cdot 1.4H_2O$) C, H, N.

5-Chloro-5,8-dideazapteroic Acid (11). To a suspension of 6-[(*p*-carboxyanilino)methyl]-5-chloro-2,4-diaminoquinazoline (**10**)[24] (1.25 g, 3.37 mmol) in 2-methoxyethanol (25 mL) was added 2 N HCl (25 mL), and the resulting mixture was heated at reflux for 6 h. After cooling, 50 mL of H_2O was added together with 2 N NaOH until the pH reached 11.5. Insoluble material was removed by filtration and the filtrate acidified to pH 5.9 with 1 N HCl. After refrigeration, the product was collected on a filter and washed with H_2O. It was dried under vacuum first at 45 °C for 12 h and then at 100 °C over P_2O_5 for 20 h to yield 1.08 g (91%) of pale yellow solid: mp 251–253 °C dec; TLC R_f 0.69 (Baker silica gel 1B2-F, *n*-BuOH–HOAc–H_2O, 3:1:1); NMR (Me_2SO-d_6) δ 4.37 (app d, 2, CH_2N), 6.55 (d, 2, 3′, 5′, J_0 = 8.76 Hz), 6.60 (s, 2, 2-NH_2), 6.96 (app t, 1, CH_2NH), 7.10 (d, 1, H_8, $J_{8,7}$ = 8.52 Hz), 7.46 (d, 1, H_7, $J_{7,8}$ = 8.58 Hz), 7.66 (d, 2, 2′, 6′, J_0 = 8.64 Hz). Anal. ($C_{16}H_{13}ClN_4O_3 \cdot 0.5H_2O$) C, H, N.

5-Chloro-10-(trifluoroacetyl)-5,8-dideazapteroic Acid (12a). A sample of **11** (0.84 g, 2.37 mmol) (redried under vacuum at 100 °C just prior to use) in $(CF_3CO)_2O$ (25 mL) was stirred in a N_2 atmosphere for 24 h. The mixture was concentrated under vacuum, and then Et_2O (35 mL) was added and the solution again evaporated at reduced pressure. The resulting foam was triturated with 1% aqueous CF_3COOH and the resulting solid collected on a filter, washed with H_2O, and dried under vacuum at 50 °C for 12 h to afford 1.09 g (92%) of cream-colored solid: mp 204–206 °C dec (preliminary softening); TLC R_f 0.59; NMR (Me_2SO-d_6) δ 5.14 (s, 2, CH_2N), 6.86–7.60 (m, 6, 2-NH_2, 3′, 5′, H_7 and H_8), 7.89 (d, 2, 2′, 6′, J_0 = 8.20 Hz); FAB/MS 441 (M + 1). Anal. ($C_{18}H_{12}ClF_3N_4O_4 \cdot 0.4CF_3COOH \cdot 0.6H_2O$) C, H, N.

6-[[*p*-Carboxy-*N*-(trifluoroacetyl)anilino]methyl]-5-chloro-2,4-diaminoquinazoline (12b). A mixture of **10** (0.50 g, 1.35 mmol) and $(CF_3CO)_2O$ (15 mL) was stirred in a N_2 atmosphere for 7 h. The mixture was evaporated under reduced pressure and the resulting foam triturated with 2 × 20 mL of 1% aqueous CF_3COOH and then Et_2O (15 mL). The resulting solid was isolated by filtration, washed with Et_2O, and dried under vacuum at 45 °C to yield 0.33 g of pale yellow solid, mp 304–306 °C dec. The combined Et_2O washings and filtrate were evaporated to yield a second crop, which was triturated with 1% aqueous CF_3COOH, isolated by filtration, washed with H_2O, and dried as above to give an additional 0.32 g of product: total yield 0.65 g (95%); TLC R_f 0.39; NMR (Me_2SO-d_6) δ 5.15 (s, 2, CH_2N), 7.04 (br s, 2, NH_2), 7.14 (d, 1, H_7 or H_8, $J_{7,8}$ = 9.00 Hz), 7.39 (d, 2, 3′, 5′, superimposed on d for H_7 or H_8), 7.89 (d, 2, 2′, 6′, J_0 = 8.37 Hz, superimposed on br s, NH_2). Anal. ($C_{18}H_{13}ClF_3N_5O_3 \cdot 0.5C$-$F_3COOH \cdot 0.5H_2O$) C, H, N.

N^{δ}-(*tert*-Butyloxycarbonyl)-N^{α}-(5-chloro-5,8-dideazapteroyl)-L-ornithine (14a). To a stirred solution of **12a** (0.50 g, 1.01 mmol) in DMF (35 mL) at 0 °C was added Et_3N (0.2 g, 2.01 mmol), followed by *i*-BuOCOCl (0.21 g, 1.51 mmol). The solution was stirred at 0 °C under N_2 for 0.75 h, at which time **8** (0.35 g, 1.51 mmol) was added. Stirring was continued at 0 °C for 4 h, after which the solvent was removed under reduced pressure and the residual oil triturated with H_2O. The solid was isolated by filtration, washed with H_2O, and dried overnight under vacuum at 45 °C to give 0.76 g of pale yellow solid. The TLC ($CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5) showed two spots: a major one (R_f 0.16), presumably **13a**, and a minor spot (R_f 0.08), which corresponded to **12a**. A solution of crude **13a** (0.76 g) in 10% NH_4OH (100 mL) was stirred at ambient temperature for 1.25 h. The solvent was removed under reduced pressure to give 0.75 g of crude **14a**, which was purified in two batches on a silica gel column (17 × 1.9 cm) packed in $CHCl_3$ and eluted with $CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5. Fractions homogeneous by TLC were pooled and evaporated to dryness at reduced pressure. The residue was triturated with 3 × 15 mL of Et_2O and the resulting solid dried under vacuum over P_2O_5 at 65 °C for 8 h. There was obtained 0.34 g (52% overall yield) of greenish yellow solid: mp 115–120 °C dec (preliminary softening); TLC R_f ($CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5); NMR (Me_2SO-d_6) δ 1.20–1.88 [m, 13, CH_2CH_2 and $C(CH_3)_3$], 2.89 (m, 2, CH_2NHCOO), 4.19 (m, 1, orn α-CH), 4.38 (app d, 2, CH_2NH, J = 5.79 Hz), 6.55 (d, 4, 3′, 5′, J_0 = 8.70 Hz, superimposed upon br s, 2-NH_2), 6.80 (m, 2, CH_2NH and NHCOO), 7.09 (d, 1, H_8, $J_{8,7}$ = 8.55 Hz), 7.46 (d, 1, H_7, $J_{7,8}$ = 8.64 Hz), 7.62 (d, 2, 2′, 6′, J_0 = 8.76 Hz), 7.92 (d, 1, CONH, J = 6.84 Hz). Anal. ($C_{26}H_{31}ClN_6O_6 \cdot 5.25H_2O$) C, N; H: calcd, 6.40; found, 5.12. (Reproducible combustion analyses could not be obtained.)

N^{α}-(5-Chloro-5,8-dideazapteroyl)-L-ornithine (3e). A solution of **14a** (0.20 g, 0.31 mmol) in CF_3COOH (6 mL) was stirred at ambient temperature for 1 h. The CF_3COOH was removed under reduced pressure and the residue triturated with 3 × 15 mL of Et_2O. After drying, the solid was dissolved in 10% NH_4OH (15 mL) and the solution evaporated to dryness under vacuum. Residual NH_3 was removed by entrainment with 3 × 30 mL of H_2O and 20 mL of EtOH. The residue was triturated with cold EtOH and the resulting solid isolated by filtration, washed with cold EtOH and Et_2O, and dried under vacuum at 45 °C for 15 h and then over P_2O_5 at 65 °C for 8 h to give 0.11 g (58%) of greenish yellow solid: mp 194.5–199 °C dec; TLC R_f 0.22; UV

λ_{max} 234 (ϵ 38.9 × 10^3), 292 (22.7 × 10^3) nm; NMR (CF_3COOD) δ 2.12–2.54 (two br s, 4, CH_2CH_2), 3.51 (br s, 2, CH_2NH_2), 5.14 (s, 1, orn α-CH), 5.21 (s, 2, CH_2NH), 7.03 (br s), 7.58 (d, 1, H_8, $J_{8,7}$ = 8.70 Hz), 7.83 (d, 2, 3′, 5′, J_0 = 7.80 Hz), 8.04 (d, 1, H_7, $J_{7,8}$ = 8.10 Hz), 8.17 (d, 2, 2′, 6′, J_0 = 9.00 Hz); FAB/MS 459 (M + 1), 457 (M − 1). Anal. ($C_{21}H_{23}ClN_6O_4 \cdot CF_3COOH \cdot H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-5-chloro-5,8-dideazapteroyl)-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithine (14b). The N^{10}-(trifluoroacetyl) derivative **13b** was prepared from **12b** and **8** in an analogous fashion to that employed for **13a**. The crude product showed two major spots on TLC ($CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5) with R_f values of 0.32 (**13b**) and 0.21 (**12b**). A solution of crude **13b** (0.60 g) in 10% NH_4OH (100 mL) was stirred for 1.25 h and then worked up the same as for the preparation of **14a** to give 0.15 g (28% overall yield) of a pale yellow solid: mp 178.5–180.5 °C dec; TLC R_f 0.25 ($CHCl_3$–MeOH–NH_4OH, 7:2.5:0.5); NMR (Me_2SO-d_6) δ 1.22–1.90 [m, 13, CH_2CH_2 and $C(CH_3)_3$], 2.91 (app q, 2, CH_2NHCOO, J = 5.79 Hz), 4.26 (m, 1, orn α-CH), 4.38 (app d, 2, CH_2NH, J = 5.13 Hz), 6.18 (s, 2, NH_2), 6.57 (d, 2, 3′, 5′, J_0 = 8.64 Hz), 6.82 (m, 2, CH_2NH and NHCOO), 7.14 (d, 1, H_7 or H_8, $J_{7,8}$ = 8.70 Hz), 7.44 (d, 3, H_7 or H_8, $J_{7,8}$ = 8.70 Hz, superimposed upon s, NH_2), 7.65 (d, 2, 2′, 6′, J_0 = 8.64 Hz), 8.05 (d, 1, CONH, J = 8.04 Hz). Anal. ($C_{26}H_{32}ClN_7O_5 \cdot 1.55H_2O$) C, H; N: calcd, 16.73; found, 16.14.

N^{α}-(4-Amino-4-deoxy-5-chloro-5,8-dideazapteroyl)-L-ornithine (3f). This compound was prepared in a similar manner as **3e** starting with 0.10 g (0.17 mmol) of **14b**. There was obtained 52 mg (49%) of yellow solid: mp 174.5–176.5 °C dec; TLC R_f 0.15; UV λ_{max} 240 (ϵ 33.4 × 10^3), 286 (21.9 × 10^3) nm; NMR (Me_2SO-d_6) δ 1.46–2.00 (m, 4, CH_2CH_2), 2.79 (m, 2, CH_2NH_2), 4.12–4.56 (m, 3, CH_2NH and orn α-CH), 6.27 (s, 2, NH_2), 6.58 (d, 2, 3′, 5′, J_0 = 7.98 Hz), 6.86 (s, 1, CH_2NH), 7.14 (d, 1, H_7 or H_8, $J_{7,8}$ = 9.03 Hz), 7.45 (d, 1, H_7 or H_8, $J_{7,8}$ = 9.00 Hz), 7.50 (s, 2, NH_2), 7.65 (d, 2, 2′, 6′, J_0 = 8.22 Hz), 8.10 (d, 1, CONH, J = 9.00 Hz); FAB/MS 458 (M + 1). Anal. ($C_{21}H_{24}ClN_7O_3 \cdot 1.25CF_3COOH \cdot H_2O$) C, H, N.

Di-*tert*-butyl-5-chloro-5,8-dideazafolate (16). To a stirred solution of **12a** (0.135 g, 0.27 mmol) and di-*tert*-butyl-L-glutamate (0.082 g, 0.28 mmol) in anhydrous DMF (20 mL) at 0 °C was added Et_3N (0.051 g, 0.5 mmol) in DMF (2 mL) followed by immediate addition of *i*-Bu-OCOCl (0.069 g, 0.5 mmol) in DMF (2 mL). The solution was stirred at 0 °C under N_2 for 2 h, at which time TLC of the reaction mixture showed the presence of the product as well as unreacted **12a**; however, there was no trace of di-*tert*-butyl-L-glutamate in the reaction mixture. Consequently, di-*tert*-butyl-L-glutamate (0.05 g, 0.169 mmol) was added to the reaction mixture followed by subsequent addition of *i*-Bu-OCOCl (0.05 g, 0.36 mmol) and Et_3N (0.05 g, 0.49 mmol). Stirring was continued at 0 °C for 2 h, followed by 16-h stirring at ambient temperature, after which the solvent was removed under reduced pressure and the residual brownish oil was washed three times with Et_2O and then triturated with H_2O. A pale yellow solid was isolated by filtration, washed with H_2O, and dried under vacuum at 50 °C to give 0.115 g (62.5%) of crude **15**. TLC ($CHCl_3$–MeOH, 85:15) of the product showed two spots: a major UV-absorbing spot (R_f 0.51) presumably due to **15** and a minor spot near the origin, which corresponded to **12a**. A solution of crude **15** (0.115 g) in 10% ethanolic NH_4OH (50 mL) was stirred at ambient temperature for 18 h. The solvent was removed under reduced pressure, and residual traces of NH_3 and H_2O were removed by entrainment with EtOH and then with Et_2O. There was obtained a yellow solid, which was dissolved in a mixture of $CHCl_3$ (20 mL) and MeOH (1 mL), and the resulting solution was filtered to remove insoluble yellow material. The clear filtrate was evaporated to dryness under vacuum to give 0.103 g (52% overall yield) of a light yellow solid, **16**, which was purified on a silica gel column (15 × 1.9 cm) packed in $CHCl_3$ and eluted with $CHCl_3$–MeOH, 95:5. Fractions homogeneous by TLC were pooled and evaporated to dryness under reduced pressure to give 0.071 g of a pale yellow solid: mp 167–168 °C dec; TLC ($CHCl_3$–MeOH, 9:1) R_f 0.27; NMR (Me_2SO-d_6, 400 MHz) δ 1.36 [s, 9, $C(CH_3)_3$], 1.38 [s, 9, $C(CH_3)_3$], 1.82–2.02 (m, 2, glu-β-CH_2), 2.28 (t, 2, glu-γ-CH_2, J = 14.96 Hz), 4.23–4.29 (m, 1, glu-α-CH), 4.37 (d, 2, CH_2NH, J = 4.84 Hz), 6.38 (br s, 2, 2-NH_2), 6.54 (d, 2, 3′, 5′, J_0 = 8.76 Hz), 6.80 (t, 1, CH_2NH, J = 11.92 Hz), 7.08 (d, 1, H_8, $J_{8,7}$ = 8.58 Hz), 7.45 (d, 1, H_7, $J_{7,8}$ = 8.56 Hz), 7.63 (d, 2, 2′, 6′, J_0 = 8.76 Hz), 8.07 (d, 1, CONH, J = 7.64 Hz). This compound was not further characterized but was used directly for the preparation of **2e**.

5-Chloro-5,8-dideazafolic Acid (2e). A solution of **16** (0.05 g, 0.085 mmol) in CF_3COOH (2 mL) was stirred at ambient temperature for 4 h. The CF_3COOH was removed under reduced pressure and the residue triturated with 3 × 5 mL of Et_2O and dried. The compound was purified by dissolving it in 0.1 N NaOH (5 mL) and stirring it with cellulose (EM Sciences/2331) (0.03 g) and charcoal (0.01 g) for 2 h. The solution was filtered by gravity and product precipitated from the clear filtrate by adding 1 N HCl and adjusting the pH to 3.5. The product was isolated by filtration, dried, and purified by applying to a cellulose column packed in 5% NH_4HCO_3 and eluted with 5% NH_4HCO_3. Fractions homogeneous by TLC were pooled and evaporated under vacuum. The residue was dissolved in H_2O (5 mL) and the solution acidified to pH 3.5 with 1 N HCl. After the solution was cooled at 0 °C for 2 h, the white solid was collected by filtration and washed with cold H_2O, Me_2CO, and finally with Et_2O. The solid was dried under vacuum at 60 °C for 8 h to give 0.019 g (47%) of **2e**. The overall yield from **12a** was 24.5%: mp >232 °C dec (with preliminary darkening and shrinking) (lit.[26] mp >230 °C dec); TLC (cellulose 5% NH_4HCO_3), R_f 0.34; UV λ_{max} 234 (ϵ 38.0 × 10^3), 286 (ϵ 22.6 × 10^3) nm; UV spectrum of **2e** in 0.1 N NaOH was in good agreement with the literature values;[26] NMR (Me_2SO-d_6, 400 MHz) δ 1.82–2.10 (m, 2, glu-β-CH_2), 2.30 (t, 2, glu-γ-CH_2, J = 14.96 Hz), 4.29–4.34 (m, 1, glu-α-CH), 4.37 (d, 2, CH_2NH, J = 5.76 Hz), 6.38 (br s, 2, 2-NH_2), 6.55 (d, 2, 3′, 5′, J_0 = 8.8 Hz), 6.80 (t, 1, CH_2NH, J = 11.8 Hz), 7.08 (d, 1, H_8, $J_{8,7}$ = 8.56 Hz), 7.44 (d, 1, H_7, $J_{7,8}$ = 8.56 Hz), 7.64 (d, 2, 2′, 6′, J_0 = 8.76 Hz), 8.09 (d, 1, CONH, J = 7.72 Hz); FAB/MS 474 (M + 1). Anal. ($C_{21}H_{20}ClN_5O_6 \cdot 2.75H_2O$) C, H, N.

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Registry No. **2a**, 118895-96-8; **2c**, 118895-97-9; **2e**, 74671-08-2; **2f**, 20242-62-0; **3a** (free base), 120296-43-7; **3b** (free base), 120296-44-8; **3c** (free base), 120296-45-9; **3c**·TFA, 120296-46-0; **3d** (free base), 120296-47-1; **3d**·TFA, 120296-48-2; **3e** (free base), 120296-49-3; **3e**·TFA, 120296-50-6; **3f** (free base), 120296-51-7; **3f**·TFA, 120296-52-8; **4**, 106585-53-9; **6**, 120296-53-9; **7a**, 120296-54-0; **7b**, 111718-85-5; **7c**, 118895-93-5; **7d**, 64088-70-6; **8**, 13650-49-2; **9a**, 120296-55-1; **9b**, 120296-56-2; **9c**, 120296-57-3; **9d**, 120296-58-4; **10**, 57343-54-1; **11**, 120296-59-5; **12a**, 120296-60-8; **12b**, 120296-61-9; **13a**, 120296-62-0; **13b**, 120296-63-1; **14a**, 120296-64-2; **14b**, 120296-65-3; **15**, 120296-66-4; **16**, 120296-67-5; FPGS, 63363-84-8; DHFR, 9002-03-3; 4,4′-$EtOCOC_6H_4SSC_6H_4COOEt$, 20057-83-4; $(CF_3CO)_2O$, 407-25-0; H-Glu(OBu-*t*)-OBu-*t*, 16874-06-9.