Synthesis and Biological Evaluation of *N*α-(4-Amino-4-deoxy-10-methylpteroyl)-DL-4,4-difluoroornithine

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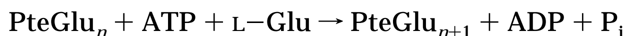
*N*α-(4-Amino-4-deoxy-10-methylpteroyl)-DL-4,4-difluoroornithine (AMPte-DL-4,4-F_2Orn, **4**) was synthesized and evaluated as an inhibitor of human folylpoly-γ-glutamate synthetase (FPGS), dihydrofolate reductase (DHFR), and cell growth. Synthesis of **4** involved the use of a protected form of DL-4,4-difluoroornithine **9** which was derived from DL-4,4-difluoroglutamic acid. Biological activities of **4** were compared directly to those of the corresponding nonfluorinated compound *N*α-(4-amino-4-deoxy-10-methylpteroyl)-L-ornithine (AMPte-L-Orn, **3**). Although the fluorinated analogue is a potent inhibitor of DHFR, it is a poor inhibitor of FPGS. However, the compound is transported across the cell membrane and inhibits cell growth, presumably due to the inhibition of DHFR. The data obtained with the fluorinated analogue are in contrast to those of the corresponding nonfluorinated compound **3**, which is a potent inhibitor of both FPGS and DHFR but shows very low cytotoxicity due to poor transport.

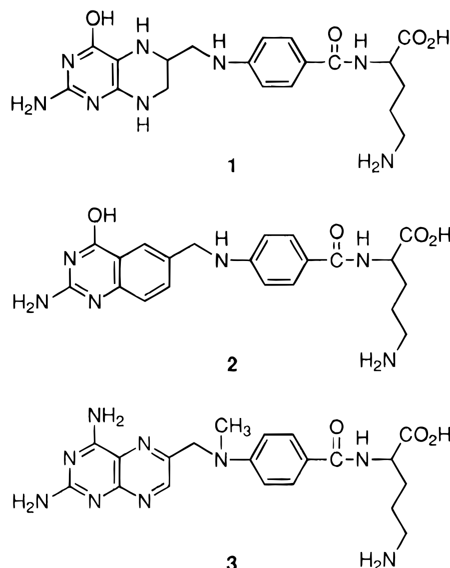
Introduction

Reduced folates play an important role as coenzymes for folate-dependent enzymes and are required for the one-carbon transfers involved in biosynthesis of certain amino acids and nucleic acid precursors.[1] Reduced folates are present in cells as poly-γ-glutamate conjugates, which are preferred substrates for most folate-dependent enzymes.[2] These poly-γ-glutamate conjugates are polyanionic compounds that do not readily cross the cell membrane and thus are well retained by cells.[3] The intracellular formation of the poly-γ-glutamate conjugates is catalyzed by an ATP-dependent enzyme, folylpoly-γ-glutamate synthetase (FPGS), as shown below.



$$\mathrm{PteGlu}_n + \mathrm{ATP} + \mathrm{L{-}Glu} \rightarrow \mathrm{PteGlu}_{n+1} + \mathrm{ADP} + \mathrm{P_i}$$

Inhibitors of FPGS could be useful agents for studying the physiological roles of folylpolyglutamates and could be effective chemotherapeutic agents. Shane and coworkers reported that *N*α-(tetrahydropteroyl)-L-ornithine (**1**) is a potent inhibitor of mammalian FPGS (K_i = 0.2 μM).[4,5] Since their pioneering work, numerous ornithine-containing analogues with modified pteroyl moieties have been synthesized and were found, in general, to be very effective inhibitors of FPGS.[6−10] The potency of the inhibitors is dependent on the nature of the pteroyl moiety. They are classified into two groups of interest: (1) specific inhibitors of FPGS, represented by a series of 5,8-dideazapteroyl (quinazoline) analogues of pteroylornithine (e.g., **2**),[8−10] and (2) so-called "dual-site" inhibitors that act against both FPGS and a second folate-dependent enzyme such as dihydrofolate reductase (DHFR). The methotrexate (MTX) analogue *N*α-(4-amino-4-deoxy-10-methylpteroyl)-L-ornithine (AMPte-L-Orn, **3**) and other 2,4-diamino derivatives[5−7,9] belong to this second class. The first class of inhibitors is of interest in connection with the long-standing question



of possible cytotoxic effect on cell growth and/or viability elicited by specific inhibition of FPGS. The second class of inhibitors is important because they may show enhanced cytotoxicity due to their two sites of action. Unfortunately, all of the ornithine-containing analogues of folate and antifolates were only weakly inhibitory to cell growth. This has been attributed to poor transport of the inhibitors across the cell membrane caused by the positively charged terminal amino group (pK_a = 10.8) of ornithine.

Poor transport might be overcome by replacing the ornithine residue in these inhibitors with 4,4-difluoroornithine (4,4-F_2Orn). This amino acid is presumed to have a neutral terminal amino group at physiological pH due to the electron-withdrawing effect of the fluorine atoms. In fact, 5,5-difluorolysine has been synthesized previously, and the basic dissociation constant of the terminal amino group was determined to be 6.88.[11] Assuming that 4,4-F_2Orn has a similar pK_a value as 5,5-difluorolysine, 4,4-F_2Orn-containing folate and anti-

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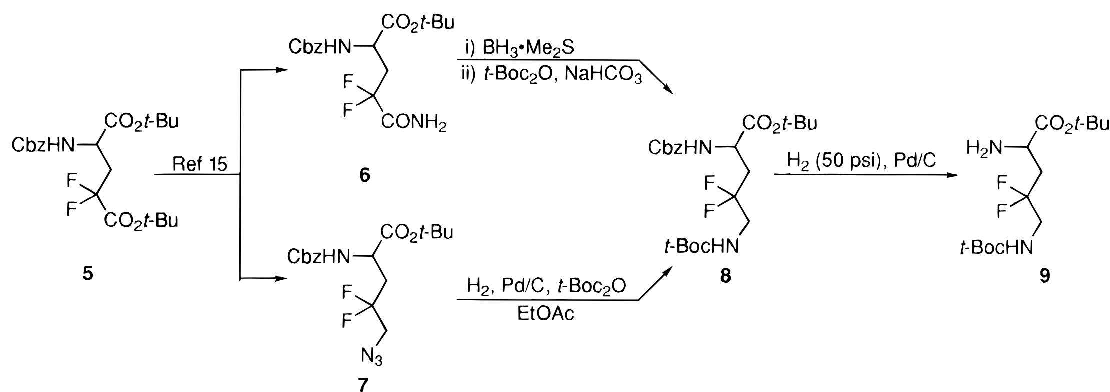
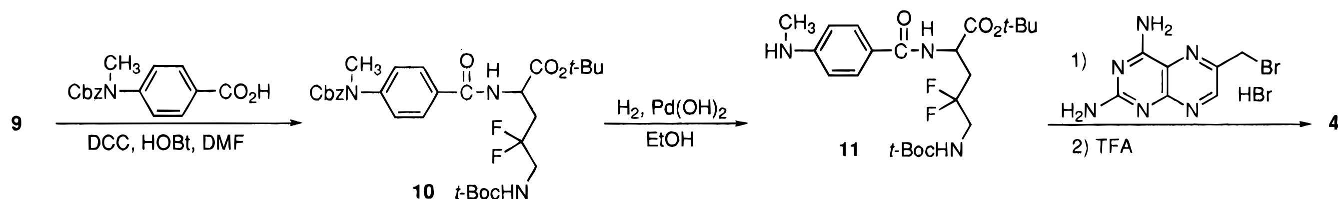
Scheme 1

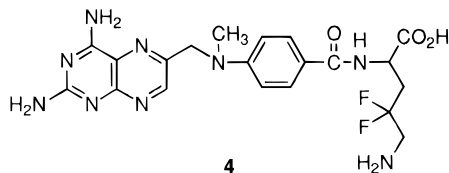


Scheme 2



folate analogues possibly could be transported more efficiently across cell membranes.

Since there is no well-defined phenotype of FPGS-inhibited cells, the biological evaluation of FPGS-specific inhibitors is not straightforward and therefore, the evaluation of transport ability of 4,4-F_2Orn-containing FPGS-specific inhibitors could be complicated. In contrast, inhibition of DHFR by dual-site inhibitors is expected to result in decreased cell growth. Therefore, 4,4-F_2Orn-containing analogues of the dual-site inhibitors could be more straightforward probes in studying the effect of fluorine substituent on the transport capability of the inhibitors. Thus, as an initial target, we have focused our effort on a potential dual-site inhibitor and have synthesized the 4,4-F_2Orn-containing methotrexate analogue N^α-(4-amino-4-deoxy-10-methylpteroyl)-DL-4,4-difluoroornithine (AMPte-DL-F_2Orn, **4**). The biological activity of this new analogue has been compared to the corresponding nonfluorinated compound **3**.



Chemistry

The synthetic studies first focused on preparation of the 4,4-difluoroornithine portion of the target molecule. Initially, we investigated DAST-mediated difluorination[12] of synthetically available L-4-oxoornithine derivatives.[13,14] Our attempts, however, proved unsuccessful as either aziridine formation or poor reactivity was found in the reactions of these substrates (see Supporting Information for details).

We found recently that N-Cbz-protected di-*tert*-butyl 4,4-difluoroglutamate **5** undergoes various regioselective nucleophilic additions at the γ-carbonyl group (Scheme 1), thus leading to a facile synthesis of amide **6** and azide **7**.[15] Attempted reduction[16] of the amide **6** using borane–dimethyl sulfide was nonselective based on a ^{19}F NMR spectrum of the crude product, which appeared to be a mixture of several fluorinated compounds, the structures of which have not been established. Subsequent derivatization of the crude amine as a *tert*-butyl carbamate afforded **8** in 33% yield (26% overall yield from **5**). Azide **7** was then examined as an alternate precursor of **8**. Treatment of the azide **7** with Ph_3P in THF–H_2O followed by protection of the terminal amino group afforded **8** in 41% yield (27% overall yield from **5**). The best yield of **8** was obtained when the azide **7** was treated with H_2 over Pd/C (1 atm) in the presence of di-*tert*-butyl dicarbonate.[17] This convenient one-pot procedure gave **8** in 82% yield (54% overall yield from **5**). It should be noted that the benzyl carbamate group of **7** remained intact during the catalytic hydrogenation. However, under H_2 at higher pressure (50 psi) the Cbz group of **8** was readily cleaved to give **9** in 87% yield. The 4,4-difluoroornithine derivative **9** is an ideal precursor for the synthesis of 4,4-difluoroornithine-containing folate and antifolate analogues for the following reasons: (1) the unprotected α-amino group favors coupling to a wide variety of pteroyl moieties, (2) the protection of both the terminal δ-amino group and α-carboxylic acid allows easy purification, if necessary, of synthetic intermediates, and (3) the *tert*-butyl ester and the *tert*-butyl carbamate can be readily cleaved together in the final step of the synthesis by treatment with TFA.

Incorporation of a pteroyl moiety into **9** has been performed according to the method reported by Piper and co-workers.[18,19] Condensation of **9** with the N-Cbz-protected derivative of N-methyl-4-aminobenzoic acid afforded **10** (93% yield), which was subsequently deprotected by catalytic hydrogenation to give **11** in 88% yield (Scheme 2). Coupling of **11** to 6-(bromomethyl)-2,4-pteridinediamine hydrobromide in dimethylacetamide followed by hydrolysis with TFA and precipitation from ether gave **4** as a yellow solid in 44% overall yield for the final two steps.

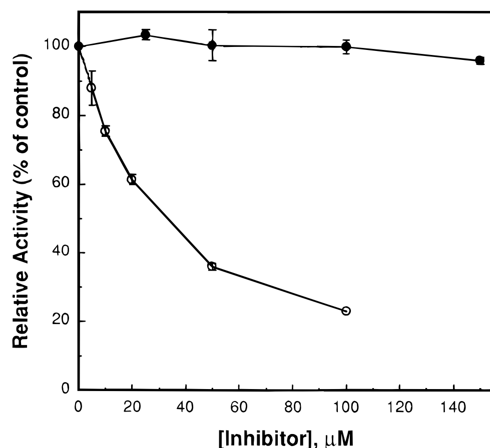


Figure 1. Inhibition of FPGS-catalyzed glutamylation of MTX by AMPte-L-Orn (**3**; ○) and AMPte-DL-4,4-F_2Orn (**4**; ●). For AMPte-DL-4,4-F_2Orn, only the concentration of L-isomer is indicated.

Table 1. Biological Evaluation of Ornithine-Containing Analogues of Methotrexate[a]

target	3	4[b]	MTX
FPGS,[c] IC_{50} (μM)	33 ± 2	>300	
DHFR,[d] IC_{50} (nM)	2.5[f]	1.3 ± 0	0.82 ± 0.04
cell growth,[e] ED_{50} (nM)	740[f]	93 ± 17	14.5 ± 0.5

[a] All experiments were done in duplicate, and data are presented as average values ± range. [b] All concentrations shown are for the L-isomer; it has been demonstrated that the D-isomer of MTX is biologically inactive.[29,30] [c] FPGS isolated from CCRF-CEM human leukemia cells.[25] [d] DHFR isolated from CCRF-CEM human leukemia cells.[25] [e] 120 h continuous exposure of CCRF-CEM human leukemia cells. [f] Literature data[7] for DHFR inhibition and cytotoxicity for MTX are as follows: DHFR IC_{50} = 1.0 nM, ED_{50} = 11 nM.

Biological Evaluation

The new MTX analogue **4** was first evaluated as an inhibitor of human FPGS. As shown in Figure 1, compound **4** was found to be devoid of inhibitory activity at concentrations as high as 150 μM. In contrast, the nonfluorinated analogue N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-L-ornithine, **3**, was confirmed as a potent inhibitor of FPGS (Figure 1). Assuming that the pK_a of the δ-amino group of ornithine in **3** is unchanged from that of the free amino acid (pK_a = 10.8), the predominant species present in solution under standard FPGS assay conditions (pH = 8.5) would be the protonated form. This would not be the case with **4,** again assuming that the pK_a of **4** is unchanged from that of the free amino acid (pK_a = ca. 6.9, *vide supra*). In order to evaluate any pH dependence of the inhibition by **4**, assays of FPGS activity were run at pH = 7.9, 8.5, and 9.0 in the presence of **4** (200 μM). There was no significant inhibition of FPGS by **4** under any of the assay conditions. It is not possible to evaluate **4** at lower pH values because control enzyme activity declines sharply below pH 7.9.

Although the lack of FPGS inhibition by **4** was disappointing, we pursued other aspects of antifolate activity (i.e., inhibition of DHFR and cell growth) in order to assess the ability of **4** to cross cell membranes and ultimately to compare the cytotoxic effects of **4** vs **3**. As shown in Table 1, compound **4** is a potent inhibitor of DHFR and comparable to both MTX and the ornithine analogue **3**. Given the potent inhibition of DHFR by **4**, this new MTX analogue was evaluated for its effect on the growth of CCRF-CEM human leukemia cells. The data in Table 1 demonstrate that **4** is more potent than is **3**. The growth and DHFR inhibitory potency data suggest that transport of the fluorinated ornithine analogue **4** into cells is considerably improved over that of the corresponding nonfluorinated species **3**.

Conclusions

In order for a molecule to affect FPGS activity intracellularly, two requirements must be satisfied as follows: (1) The molecule is efficiently transported into cells. (2) The molecule is an inhibitor of FPGS. Compound **3** meets the second requirement but not the first, while the opposite is true for compound **4**. The data presented here indicate that, as has been suggested by several research groups, poor transport of ornithine-containing analogues of folates and antifolates is due to the positively charged terminal amino group. The transport capability can be improved by reducing the basicity of the terminal amino group as clearly shown with compound **4**. However, such modification results in loss of FPGS inhibitory activity. Since the structure of any complex involved in FPGS catalysis has not been established, the molecular basis for the marked difference in the inhibition observed between **3** and **4** is unclear. One possibility is that the positively charged terminal amino moiety of **3** binds via an electrostatic interaction with an anionic residue on FPGS. This would explain the lack of FPGS inhibition observed with **4** since the neutral form is the predominant species at physiological pH. The structural requirements for transport and FPGS inhibition observed with **3** and **4** present a problem in designing ornithine-containing analogues as inhibitors of FPGS that will be effective both *in vitro* and *in vivo*. The present findings should be considered in future research on the development of FPGS inhibitors that are capable of being transported into cells.

Experimental Section

Thin layer chromatography was performed with E. Merck silica gel 60 F-254 plates. Column chromatography was performed with silica gel 60 (230−400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on either a Bruker AM-300 or AM-360 spectrometer. ^{19}F NMR spectra were obtained using a General Electric AM-500 spectrometer. Chemical shifts are in parts per million downfield from tetramethylsilane (internal standard for ^{1}H and ^{13}C) or trifluoroacetic acid (external standard for ^{19}F). Infrared spectra were obtained on a Nicolet 5-DX spectrometer. Mass spectra and high-resolution mass spectra were obtained using a Finnegan 4500 GC/MS-EICI system or a VG Analytical system, Model 70-250S. Elemental analyses were obtained from the Elemental Analysis Laboratory, Department of Chemistry, The University of Michigan, or Atlantic Microlabs, Atlanta, GA. UV spectra were obtained on a Beckman Model DU-7 spectrophotometer. High-pressure hydrogenations were carried out with a Parr hydrogenator. Compound **3** was synthesized by the method of Piper et al.[19]

***N*²-[(Benzyloxy)carbonyl]-*N*⁵-[(1,1-dimethylethoxy)carbonyl]-DL-4,4-difluoroornithine, *tert*-Butyl Ester (8).** To a solution of **7**[15] (304 mg, 0.79 mmol) and di-*tert*-butyl dicarbonate (207 mg, 0.95 mmol) in ethyl acetate (10 mL) was added 10% Pd/C (20 mg), and the suspension was stirred under H_2 (1 atm) for 30 min. The catalyst was removed by filtration, and the filtrate was concentrated. The resulting oil was purified by silica gel chromatography (hexane/EtOAc, 4/1) to give 296 mg (82% yield) of **8** as a colorless oil: R_f 0.33 (hexane/EtOAc, 4/1); IR (neat) 3353, 2981, 2938, 1736, 1694 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 18 H), 2.2−2.6 (m, 2 H), 3.4−3.7 (m,

2 H), 4.51 (dt, J = 5.6, 6.6 Hz, 1 H), 4.88 (br, 1 H), 5.13 (s, 2 H), 5.69 (d, J = 7.3 Hz, 1 H), 7.2−7.4 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 28.0 (3 C), 28.4 (3 C), 35.7 (t, J = 23.3 Hz), 45.4 (t, J = 30.3 Hz), 50.2, 67.1, 80.6, 82.6, 122.4 (t, J = 244 Hz), 128.2 (2 C), 128.3, 128.7 (2 C), 136.5, 155.9, 156.0, 170.1; ^{19}F NMR ($CDCl_3$) δ −27.0 (ddq, J = 9.5, 251, 11.8 Hz, 1 F), −25.4 (ddq, J = 7.4, 251, 13.2 Hz, 1 F); MS (CI) m/e (rel intensity) 459 (MH^+, 24.8), 420 (27.2), 403 (30.2), 393 (26.3), 364 (100), 347 (53.4), 303 (99.0); HRMS (CI) m/e calcd for $C_{22}H_{32}F_2N_2O_6H$ (MH^+) 459.2307, found 459.2322. Anal. ($C_{22}H_{32}F_2N_2O_6$·$0.5H_2O$) C, H, N.

***N*^5-[(1,1-Dimethylethoxy)carbonyl]-DL-4,4-difluoroornithine, *tert*-Butyl Ester (9).** To a solution of **8** (230 mg, 0.50 mmol) in dry ethanol (10 mL) was added 10% Pd/C (20 mg), and the mixture was shaken under H_2 (50 psi) for 11 h. Catalyst was removed by filtration, and the filtrate was concentrated. The resulting residue was purified by column chromatography (hexane/EtOAc, 1/1) to give 142 mg (87% yield) of **9** as a colorless oil: R_f 0.21 (hexane/EtOAc, 1/2); IR (neat) 3386, 2984, 2934, 1727 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9 H), 1.42 (s, 9 H), 1.63 (br, 2 H), 2.02 (ddt, J = 8.5, 18.6, 15.4 Hz, 1 H), 2.33 (dddd, J = 4.1, 12.0, 14.6, 20.6 Hz, 1 H), 3.3−3.6 (m, 3 H), 5.28 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 28.1 (3 C), 28.5 (3 C), 39.1 (t, J = 24.1 Hz), 45.5 (t, J = 29.9 Hz), 50.6, 60.5, 65.9, 80.1, 81.8, 122.6 (t, J = 243 Hz), 155.7, 173.6; ^{19}F NMR ($CDCl_3$) δ −27.2 (ddq, J = 13.3, 250, 19.3 Hz, 1 F), −24.3 (dp, J = 250, 14.6 Hz, 1 F); MS (CI) m/e (rel intensity) 325 (MH^+, 100), 269 (63.3), 251 (7.8), 225 (8.9), 213 (40.7); HRMS (CI) m/e calcd for $C_{14}H_{26}F_2N_2O_4H$ (MH^+) 325.1939, found 325.1934. Anal. ($C_{14}H_{26}F_2N_2O_4$) C, H, N.

***N*^2-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-*N*^5-[(1,1-dimethylethoxy)carbonyl]-DL-4,4-difluoroornithine, *tert*-Butyl Ester (10).** To a solution of 4-[[(benzyloxy)carbonyl]methylamino]benzoic acid[20] (122 mg, 0.43 mmol) in dry DMF (3 mL) were added DCC (121 mg, 0.59 mmol) and HOBt (105 mg, 0.78 mmol) at 0 °C, and the mixture was stirred at ambient temperature for 10 min. A solution of **9** (127 mg, 0.39 mmol) was then added to that mixture at 0 °C, and the mixture was stirred, at ambient temperature for 14 h (white solid appeared). The reaction mixture was filtered and the solvent (DMF) was removed under reduced pressure. The resulting crude oil was dissolved in a small volume of hexane/EtOAc, 1/1 (7 mL), and again filtered. The filtrate was evaporated, and the resulting oil was purified by silica gel chromatography (hexane/EtOAc, 4/1) to give 215 mg (93% yield) of the product **10** as a colorless oil; R_f 0.55 (hexane/EtOAc, 1/1); IR (neat) 3346, 2981, 2931, 1736, 1652, 1610 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9 H), 1.47 (s, 9 H), 2.4−2.7 (m, 2 H), 3.34 (s, 3 H), 3.3−3.8 (m, 2 H), 4.88 (dt, J = 4.6, 7.4 Hz, 1 H), 5.07 (t, J = 6.5 Hz, 1 H), 5.17 (s, 2 H), 7.2−7.4 (m, 7 H), 7.27 (d, J = 7.4 Hz, 1 H), 7.86 (d, J = 8.4 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 28.0 (3 C), 28.4 (3 C), 34.9 (t, J = 24.5 Hz), 37.5, 45.3 (t, J = 30.7 Hz), 49.2, 67.7, 80.7, 82.8, 122.4 (t, J = 244 Hz), 125.1 (2 C), 128.0 (2 C), 128.1, 128.2 (2 C), 128.6 (2 C), 131.0, 136.4, 146.3, 155.2, 156.2, 166.5, 170.2; ^{19}F NMR ($CDCl_3$) δ −27.0 (dm, J = 252 Hz, 1 F), −22.9 (dm, J = 252 Hz, 1 F); MS (EI) m/e (rel intensity) 591 (M^+, 0.9), 518 (0.6), 462 (4.9), 434 (9.8), 285 (34.3), 268 (36.0), 91 (100); HRMS (EI) m/e calcd for $C_{30}H_{39}F_2N_3O_7$ (M^+) 591.2756, found 591.2757. Anal. ($C_{30}H_{39}F_2N_3O_7$·$0.5H_2O$) C, H, N.

***N*^5-[(1,1-Dimethylethoxy)carbonyl]-*N*^2-[4-(methylamino)benzoyl]-DL-4,4-difluoroornithine, *tert*-Butyl Ester (11).** Palladium hydroxide on carbon (19 mg) was added to a solution of **10** (147 mg, 0.25 mmol) in dry ethanol (7 mL), and the mixture was shaken under H_2 (40 psi) for 8 h (Parr hydrogenator). The mixture was filtered, and the filtrate was evaporated to dryness. The crude product was purified by silica gel chromatography (hexane/EtOAc, 4/1) to give 99 mg of **11** (88%) as a colorless oil: R_f 0.38 (hexane/EtOAc, 1/1); IR (neat) 3360, 2931, 1729, 1645, 1609, 1518 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9 H), 1.47 (s, 9 H), 2.4−2.7 (m, 2 H), 2.86 (s, 3 H), 3.4−3.7 (m, 2 H), 4.1−4.4 (br, 1 H), 4.88 (dt, J = 5.0, 7.1 Hz, 1 H), 5.00 (t, J = 6.2 Hz, 1 H), 6.56 (d, J = 8.6 Hz, 2 H), 7.10 (d, J = 7.5 Hz, 1 H), 7.72 (d, J = 8.6 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 28.0 (3 C), 28.4 (3 C), 30.4, 35.5 (t, J = 23.9 Hz), 45.4 (t, J = 30.3 Hz), 48.9, 60.6, 80.6, 82.6, 111.5 (2 C), 121.9, 122.6 (t, J = 244 Hz), 129.2 (2 C), 152.3, 156.1, 167.2, 170.6; ^{19}F NMR ($CDCl_3$) δ −23.9 (dtt, J = 251, 12.6, 17.9 Hz, 1 F), −26.7 (ddq, J = 251, 19.3, 13.3 Hz, 1 F); MS (EI) m/e (rel intensity) 457 (M^+, 5.5), 383 (2.1), 328 (5.9), 300 (6.1), 134 (100); HRMS (EI) m/e calcd for $C_{22}H_{33}F_2N_3O_5$ (M^+) 457.2388, found 457.2391.

***N*^2-(4-Amino-4-deoxy-10-methylpteroyl)-DL-4,4-difluoroornithine (4).** 6-(Bromomethyl)-2,4-pteridinediamine hydrobromide[18] (35 mg, 0.09 mmol) was added to a solution of **11** (40 mg, 0.09 mmol) in dry DMAC (1.0 mL) at 0 °C, and the mixture was stirred at 55 °C for 6 h. After additional stirring (6 days) at ambient temperature, the solvent was removed by evaporation, and the resulting yellow oil was dissolved in a minimum amount of CH_2Cl_2/MeOH (20/1) and passed through a short silica gel column (CH_2Cl_2/MeOH, 20/1) to remove polar impurities. The purified di-*tert*-butyl ester was then dissolved in trifluoroacetic acid (2 mL), and the resulting solution was stirred at ambient temperature for 40 min. The reaction mixture was poured into dry ether (10 mL); the resulting precipitate was isolated by filtration and dried to give 28 mg of **4** (44%) as a bright-yellow solid: R_f 0.64 (EtOH/concentrated NH_4OH, 4/1); ^{1}H NMR (CD_3OD) δ 2.6−3.0 (m, 2 H), 3.27 (s, 3 H), 3.4−3.7 (m, 2 H), 4.8−5.0 (m, 1 H), 6.85 (d, J = 8.2 Hz, 2 H), 7.75 (d, J = 7.9 Hz, 2 H), 8.63 (s, 1 H); ^{13}C NMR (CD_3OD) δ 37.3 (t, J = 22.8 Hz), 39.9, 44.5 (t, J = 25.2 Hz), 48.7, 56.7, 112.8 (2 C), 122.1 (t, J = 244 Hz), 122.6, 123.5, 130.4 (2 C), 147.0, 150.5, 153.2, 153.8, 157.9, 165.0, 170.0, 173.9; ^{19}F NMR (CD_3OD) δ −29.0 (dm, J = 245 Hz, 1 F), −27.8 (dm, J = 245 Hz, 1 F); UV λ_{max} (pH = 13) 258, 303, 371; MS (FAB^+) m/e (rel intensity) 476 (MH^+, 71.2), 308 (40.8), 176 (100), 103 (91.7); HRMS (FAB^+) m/e calcd for $C_{20}H_{23}F_2N_9O_3H$ (MH^+) 476.1970, found 476.1954. Anal. ($C_{20}H_{23}F_2N_9O_3$·$2CF_3COOH$·H_2O) C, H; N: calcd, 17.47; found, 17.94. Reversed-phase HPLC:[21] t_R = 26.2 min.

Biochemical Techniques: Cell Lines. The human T-lymphoblastic leukemia cell line CCRF-CEM[22] the primary screen for drug effects and was the source of tumor enzymes. Routine culture of these lines was as described.[23] CCRF-CEM was verified to be negative for mycoplasma contamination during the course of these studies using the GenProbe test kit.

Enzymes and Assays: FPGS and DHFR from CCRF-CEM cells[22] were partially purified as described.[24] The FPGS assay method[25] uses [^{3}H]Glu to radiolabel polyglutamates of a folate-like substrate during incubation with the enzyme. The reaction mixture is applied to a DEAE-cellulose minicolumn that is washed with a buffered-NaCl solution to remove unligated [^{3}H]Glu. [^{3}H]Polyglutamate products are retained on the minicolumn during the wash and are then eluted quantitatively with acid. CCRF-CEM FPGS assay conditions[26] were modified to include 50 μg of protease-free bovine serum albumin (Miles). All reagents were made with freshly processed deionized water, stored frozen in aliquots, and used in only 1−2 experiments to avoid absorption of atmospheric CO_2 which can lead to erroneous FPGS kinetic constants.[26] DHFR activity was assayed spectrophotometrically as described.[27] Standard assays contained 100 mM Tris-HCl, pH 7.0, 150 mM KCl, 20 μM dihydrofolate, 20 mM 2-mercaptoethanol, and 50 μM NADPH. Inhibitory potency was measured by adding increasing concentrations of an antifolate to standard FPGS or DHFR assays and measuring the remaining activity.

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Supporting Information Available: Details of DAST-mediated difluorination of 4-oxoornithine derivatives (1 page). Ordering information is given on any current masthead page.

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