Synthesis and Biological Evaluation of DL-4,4-Difluoroglutamic Acid and DL-γ,γ-Difluoromethotrexate

Takashi Tsukamoto,[†,‡] Tomoya Kitazume,[‡] John J. McGuire,[§] and James K. Coward*[,†]

Departments of Medicinal Chemistry and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109, Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226, Japan, and Department of Experimental Therapeutics, The Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, New York 14263

Received July 14, 1995[⊗]

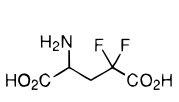
DL-4,4-Difluoroglutamic acid (DL-4,4-F_2Glu) and its methotrexate analogue, DL-γ,γ-difluoromethotrexate (DL-γ,γ-F_2MTX), were synthesized and evaluated as alternate substrates or inhibitors of folate-dependent enzymes. Synthesis of DL-4,4-F_2Glu involved the nitroaldol reaction of ethyl nitroacetate with a difluorinated aldehyde ethyl hemiacetal as a key step. Attempted ligation of DL-4,4-F_2Glu to methotrexate (MTX), catalyzed by human folylpoly-γ-glutamate synthetase (FPGS), revealed that DL-4,4-F_2Glu is a poor alternate substrate. DL-γ,γ-F_2MTX was synthesized by a route proceeding through *N*-[4-(methylamino)benzoyl]-4,4-difluoroglutamic acid di-*tert*-butyl ester followed by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide. DL-γ,γ-F_2MTX was found to be neither a substrate nor an inhibitor of human FPGS. The fluorinated analogue of MTX, however, inhibits DHFR and cell growth with the same potency as MTX.

Introduction

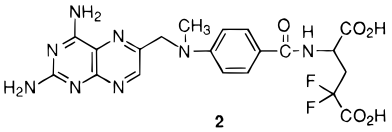
Folylpoly-γ-glutamate synthetase (FPGS) is responsible for the biosynthesis of poly-γ-glutamyl conjugates of naturally occurring folates and antifolate drugs.[1] Since folylpoly-γ-glutamates are essential for one-carbon metabolism,[2] FPGS has become an important enzyme in the study of folate biochemistry and pharmacology. The general reaction catalyzed by FPGS is shown in eq 1. In order to distinguish the glutamyl moiety on folate

$$\text{PteGlu} + \text{Glu} + \text{ATP} \rightarrow \text{PteGlu-}\gamma\text{-Glu} + \text{ADP} + \text{P}_\text{i} \quad (1)$$

(PteGlu) from free glutamic acid (Glu), it is convenient to discuss them as the accepting and incoming glutamates, respectively. In previous research from our laboratories, we have shown that, as incoming glutamates, 4-fluoroglutamic acid (4-FGlu)[3] and 3,3-difluoroglutamic acid (3,3-F_2Glu)[4] have dramatically different properties as FPGS substrates. Similarly, modification of the accepting glutamyl moiety of folates and antifolates may alter their activity as substrates or inhibitors of the FPGS-catalyzed reaction. In this regard, we have previously synthesized a series of methotrexate (MTX; 2,4-diamino-10-methylpteroyl-L-glutamic acid, AMPteGlu) analogues containing different fluoroglutamic acids. γ-Fluoromethotrexate (γ-FMTX, AMPte(4-FGlu)) was found to be polyglutamylated by FPGS at a drastically reduced rate compared to the parent molecule, MTX.[5] In contrast, enhanced substrate activity was observed with β,β-difluoromethotrexate (β,β-F_2MTX, AMPte(3,3-F_2Glu)) when compared to MTX.[6] The diametrically opposed FPGS substrate properties observed with γ-FMTX and β,β-F_2MTX are all the more striking when it is noted that both of these fluorinated MTX analogues inhibit dihydrofolate reductase (DHFR) with the same potency as does MTX. These findings prompted our interest in the synthesis of DL-4,4-difluoroglutamic acid (**1**, DL-4,4-F_2Glu) and its MTX analogue (**2**, DL-γ,γ-F_2MTX, AMPte(4,4-F_2Glu)) for further investigation of the effects of fluorine on biochemical reactions catalyzed by folate-dependent enzymes and on cell growth.[7]



1



2

Chemistry

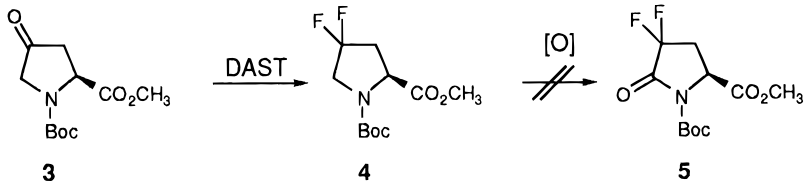
A stereospecific synthesis of L-4,4-F_2Glu has been reported previously by Kitagawa and co-workers.[8] Their method, however, requires a commercially unavailable starting material, methyl difluoroiodoacetate, which is costly to prepare.[9] In previous work, we successfully synthesized 3,3-F_2Glu from a 3-oxoproline derivative via (diethylamido)sulfur trifluoride (DAST)-mediated fluorination.[10] A similar pathway utilizing an L-4-oxoproline derivative should afford L-4,4-F_2Glu. To this end, we found that the 4-oxoproline derivative, **3**, is easily converted into the corresponding difluorinated molecule (**4**) by DAST-mediated fluorination (Scheme 1).[11] Oxidation of **4**, however, was extremely sluggish and the desired pyroglutamic acid derivative **5** was not obtained on extended (up to 10 days) reaction with RuO_4, presumably as a result of the strong electron-withdrawing effect of the two adjacent fluorine atoms (B. P. Hart and J. K. Coward, unpublished results).

These results led us to investigate a more accessible synthesis of DL-4,4-F_2Glu (**1**) by an alternate route. The difluorinated aldehyde ethyl hemiacetal, **6**, has been found previously to react with nitroalkanes to give nitro alcohols in good yields.[12] Reaction of **6** with ethyl nitroacetate in the presence of diethylamine[13] followed by acid treatment afforded nitro alcohol **7** as a mixture of diastereomers which was immediately acetylated with acetic anhydride (Scheme 2). Due to the instability of nitro alcohol **7** and the corresponding acetate, **8**, these

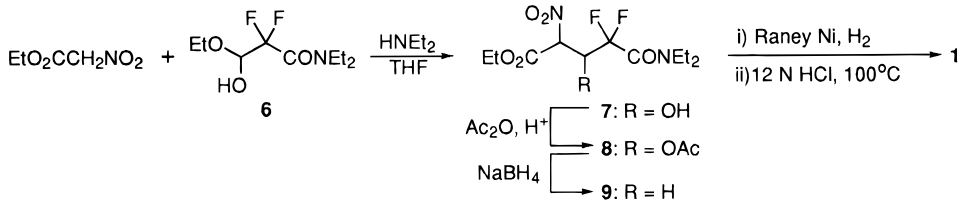
[†] The University of Michigan.
[‡] Tokyo Institute of Technology.
[§] Roswell Park Cancer Institute.
[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.

0022-2623/96/1839-0066$12.00/0

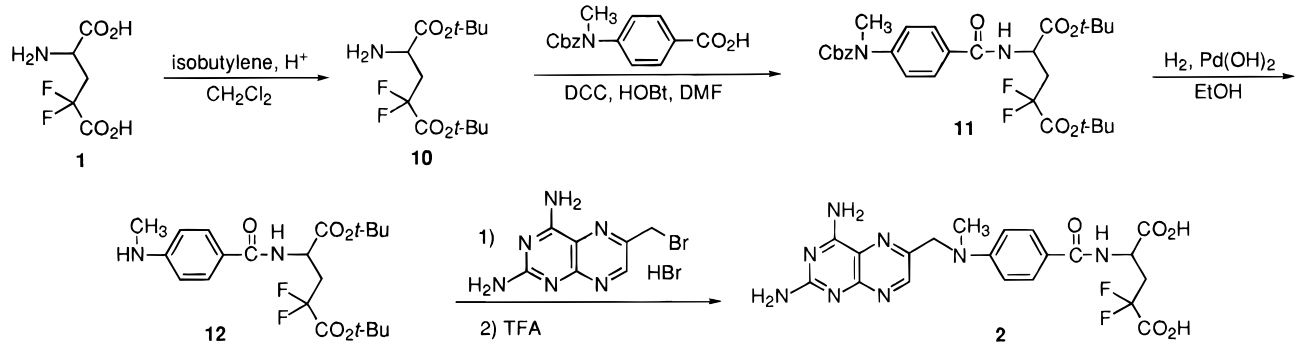
Scheme 1



Scheme 2



Scheme 3



intermediates were carried through the synthesis in crude form. Acetate **8** was treated with sodium borohydride in dry THF[14] to give the deoxygenated product **9**, a key amino acid precursor. This compound was readily purified by silica gel chromatography to afford pure material in 68% overall yield from **6**. Raney Ni-catalyzed hydrogenation followed by hydrolysis and anion-exchange chromatography gave DL-4,4-F_2Glu, **1**, in 52% overall yield from **9**. The fluorination of ketones with DAST has been a leading method for the synthesis of difluoromethylene-containing compounds. However, in contrast to the reaction with alcohols, DAST-mediated fluorination of ketones generally requires higher temperatures and longer reaction times and often gives low yields of the desired product. The synthesis described here relies on the use of a small molecule already bearing the difluoromethylene group as a building block. This methodology offers an alternate route to various difluoromethylene-containing compounds that cannot be obtained through the DAST approach.

Synthesis of DL-γ,γ-F_2MTX (**2**) was accomplished by the method reported for the synthesis of MTX by Piper and co-workers (Scheme 3).[15] DL-4,4-F_2Glu (**1**) was first converted to the corresponding di-*tert*-butyl ester, **10**, by acid-catalyzed reaction with isobutylene. Condensation of **10** with an *N*-Cbz-protected derivative of *p*-(*N*-methylamino)benzoic acid afforded *p*-(*N*-Cbz-*N*-methylamino)benzoyl 4,4-difluoroglutamate (**11**), which was subsequently deprotected by catalytic hydrogenation to give *N*-[4-(methylamino)benzoyl]-4,4-difluoroglutamic acid di-*tert*-butyl ester (**12**). Coupling of **12** and 6-(bromomethyl)-2,4-pteridinediamine hydrobromide in dimethylacetamide followed by treatment with trifluoroacetic acid afforded crude DL-γ,γ-F_2MTX (**2**). Subsequent DEAE-cellulose anion-exchange chromatography provided pure DL-γ,γ-F_2MTX (**2**) as the triethylammonium salt in 61% yield from **12**.

Biological Studies

DL-4,4-F_2Glu As the "Incoming" Glutamate. The effect of DL-4,4-F_2Glu on the FPGS-catalyzed ligation of [^{3}H]Glu to the γ-COOH of MTX was compared directly to the effect of L-*t*-4-FGlu, DL-3,3-F_2Glu, and L-Glu (isotopic dilution) (Figure 1).[3,4] DL-4,4-F_2Glu showed only slight inhibition of this FPGS-catalyzed reaction. The results indicate that DL-4,4-F_2Glu is either a poor alternate substrate or weak reversible inhibitor of FPGS. To evaluate whether DL-4,4-F_2Glu is a poor alternate substrate that can be ligated to MTX, [^{3}H]MTX was employed as the pteroyl substrate with either Glu or DL-4,4-F_2Glu as incoming the amino acid. The products formed from the FPGS-catalyzed reactions were separated by HPLC and quantitated (Table 1).[16−18] These data show that, whereas 28% of [^{3}H]MTX was converted to the γ-glutamyl dipeptide in the presence of L-Glu (higher polyglutamylated derivatives were not detected), only 5% of [^{3}H]MTX was converted into a glutamylated product in the presence of DL-4,4-F_2Glu. The product of FPGS-catalyzed ligation of DL-4,4-F_2Glu to [^{3}H]MTX is postulated to be AMPteGlu-γ-(4,4-F_2Glu) on the basis of its retention time of 33.5 min (AMPteGlu-γ-Glu, t_R = 34.5 min). These results lead to the conclusion that, although considerably less active than Glu, DL-4,4-F_2Glu also serves as an alternate incoming substrate for FPGS-catalyzed polyglutamylation. This is in marked contrast to the results obtained with a closely related *mono*-4-fluoroglutamate, L-*threo*-4-FGlu, an excellent FPGS alternate incoming substrate, the

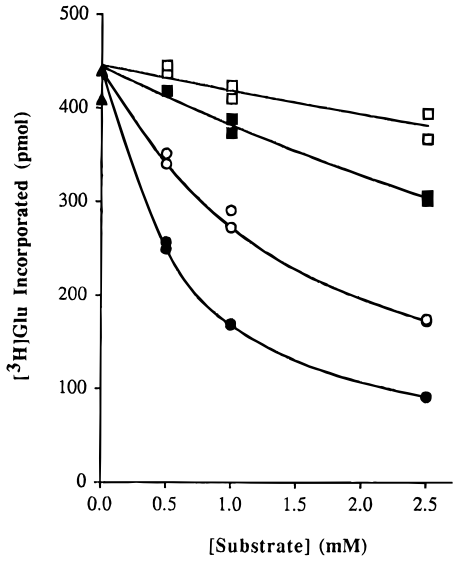


Figure 1. Effects of fluorinated glutamate analogues on the ligation of [^{3}H]Glu into methotrexate by human FPGS. CCRF-CEM FPGS was incubated with 0.5 mM L-[^{3}H]Glu (8.2 dpm/pmol) in the absence (▲) or presence of unlabeled DL-4,4-F_2Glu (□), L-*threo*-4-FGlu (■), DL-3,3-F_2Glu (●), or L-Glu (○) at the indicated concentration for 3 h. For racemic substrates, only the concentrations of L-isomers are indicated.

incorporation of which into reduced folate or MTX polyglutamates leads to termination of the ligation process.[3]

DL-4,4-F_2Glu as the "Accepting" Glutamate. The ability of DL-γ,γ-F_2MTX, the DL-4,4-F_2Glu-containing derivative of MTX, to act as an FPGS substrate was compared directly to the substrate activity of MTX and aminopterin (AMT). As shown in Table 2, no detectable [^{3}H]Glu was incorporated into DL-γ,γ-F_2MTX under conditions where ca. 400 pmol of Glu was incorporated into MTX. Since inhibitors of FPGS could potentially be useful as cytotoxic agents,[19−21]attention was focused on DL-γ,γ-F_2MTX for its ability to act as a FPGS inhibitor. DL-γ,γ-F_2MTX showed only very weak inhibition of FPGS-catalyzed incorporation of [^{3}H]Glu into AMT (IC_{50} > 200 μM, Table 3). Taken together, these results indicate that DL-γ,γ-F_2MTX binds only weakly to FPGS and is not effective as either a substrate or an inhibitor of the enzyme.

Similar to DL-γ,γ-F_2MTX, neither the *erythro* nor the *threo* isomer of DL-γ-FMTX form significant amounts of polyglutamate products.[5] In addition, neither the γ-FMTX isomers nor DL-γ,γ-F_2MTX are inhibitors of FPGS, indicating that they do not bind to the protein. In contrast, our recent studies on β,β-F_2MTX have shown that this compound is a better FPGS substrate than MTX.[6] The original hypothesis that led to the synthesis of γ-FMTX focused on the effects of the fluoro substituents on the pK_a of the γ-COOH of the accepting glutamate.[3] However, our more recent findings are inconsistent with a simple pK_a effect since the γ-COOH pK_a's of both β,β-F_2MTX and γ-FMTX are quite similar (ca. 2.5). Further studies on the mechanism of binding of the glutamyl moiety to FPGS are required before an explanation of the observed differences in reactivity between different fluoroglutamate-containing folate or antifolate substrates can be provided.

Enzyme Inhibitory Effects of DL-γ,γ-F_2MTX. In addition to FPGS substrate activity, inhibition of the target enzyme DHFR and uptake by intact cells are two biochemical properties critical to the mechanism of action of MTX analogues. Inhibition of human DHFR by DL-γ,γ-F_2MTX was compared to that observed with L-MTX (Table 4). Assuming, as shown with MTX[22] and γ-FMTX,[23] that only the L-isomer of DL-γ,γ-F_2MTX acts as an inhibitor, its inhibitory activity is nearly equivalent to that obtained from L-MTX. DL-γ,γ-F_2MTX inhibits the initial velocity of uptake of 2 μM [^{3}H]MTX by CCRF-CEM cells more potently than L-MTX itself (Table 4), suggesting that DL-γ,γ-F_2MTX has high affinity for the reduced folate/MTX carrier. These properties are similar to those observed with DL-β,β-F_2MTX.[6]

Growth Inhibitory Effects of DL-γ,γ-F_2MTX. MTX and DL-γ,γ-F_2MTX are similarly potent (based on L-isomers)[22,23] as inhibitors of growth of the CCRF-CEM human leukemia cell line during continuous exposure (Table 5). MTX-resistant sublines of CCRF-CEM having elevated DHFR activity[24] or decreased MTX uptake[25] are cross-resistant to DL-γ,γ-F_2MTX in continuous exposure (Table 5). The cross-resistance of the former subline, together with the DHFR affinity data (Table 4), suggest that DHFR is the critical intracellular target of DL-γ,γ-F_2MTX. The cross-resistance of the latter subline together with the [^{3}H]MTX uptake inhibition data (Table 4) suggest that DL-γ,γ-F_2MTX uses the reduced folate/MTX carrier system for uptake and does so with at least as high an affinity as does MTX. L-MTX and D,L-4,4-F_2MTX show similar potency against these two sublines individually instead of the ~2-fold difference observed with the parental CCRF-CEM. This may occur because, at the much higher drug concentrations required for growth inhibition of resistant lines, the D-isomer may enter cells by an alternate pathway in sufficient concentration so that it contributes to growth inhibition despite its weaker DHFR inhibition. A CCRF-CEM subline resistant to short-term, but not continuous, MTX exposure because of reduced FPGS activity[26] is not cross-resistant to continuous exposure to DL-γ,γ-F_2MTX (Table 5), indicating that, as previously shown,[5] polyglutamyl metabolites are not required during continuous exposure to potent DHFR inhibitors.

Conclusion

The data presented in this paper show that 4,4-F_2-Glu (**1**), as either an incoming or accepting glutamate, is a poor alternate substrate for the FPGS-catalyzed reaction (eq 1). When activity as an incoming glutamate is assayed directly by ligation to [^{3}H]MTX (Table 1), 4,4-F_2Glu leads to only ca. 5% ligation compared to 28% with Glu itself. The poor substrate activity may result at least partially from weak binding to FPGS, as measured indirectly by inhibition of [^{3}H]Glu incorporation (Figure 1). In previous papers from our laboratories exploring the incoming glutamate, we have described the action of *threo*-4-FGlu as a chain-terminating alternate substrate[3] and 3,3-F_2Glu as an elongation-enhancing alternate substrate.[4] Both of these analogues have higher apparent affinity for FPGS than does 4,4-F_2Glu (Figure 1). As the accepting glutamate in the MTX analogue γ,γ-F_2MTX (**2**), the presence of 4,4-F_2-Glu also leads to a poor FPGS ligand as either a substrate (Table 2) or inhibitor (Table 3). In addition to the synthesis of DL-γ,γ-F_2MTX described here, we have accomplished the synthesis of three other fluorinated analogues of MTX: *threo*-γ-FMTX, *erythro*-γ-

Table 1. HPLC Analysis of FPGS-Catalyzed Polyglutamylation of [^{3}H]MTX with L-Glu or DL-4,4-F_2Glu[a]

substrate	MTX (AMPteGlu), % (t_R = 39.2 min)	AMPteGlu-γ-Glu, % (t_R = 34.5 min)	AMPteGlu-γ-4,4F_2Glu, % (t_R = 33.5 min)
L-Glu	72	28	
DL-4,4-F_2Glu	95		5

[a] FPGS, isolated from CCRF-CEM human leukemia cells, was incubated with 0.1 μM [^{3}H]MTX (433 dpm/pmol) in the presence of L-Glu (4 mM) or DL-4,4-F_2Glu (8 mM) for 8 h under otherwise standard conditions. At the end of the incubation, samples were processed[16,18] and analyzed by reversed-phase HPLC. Fractions (1 mL, 1 min) were collected, and the radioactivity was quantitated. Sufficient chemically synthesized MTX polyglutamates to be observable by A_{280} were included as internal standards.

Table 2. FPGS-Catalyzed Polyglutamylation of Antifolates[a]

substrates	[^{3}H]Glu incorporated (pmol)
L-AMT (100 μM)	1386, −
L-MTX (100 μM)	451, 364
DL-γ,γ-F_2MTX (200 μM)[b]	2, 0

[a] CCRF-CEM FPGS was incubated for 3 h, under otherwise standard conditions, with 0.5 mM L-[^{3}H]Glu (8.2 dpm/pmol) in the presence of the indicated concentration of each antifolate. The data are single points from two independent experiments. [b] DL-γ,γ-F_2MTX concentration is twice that of other antifolates, since only the L-isomer of DL-γ,γ-F_2MTX is presumed to act as a substrate.[22,23]

Table 3. Effect of DL-γ,γ-F_2MTX on the FPGS-Catalyzed Polyglutamylation of L-Aminopterin[a]

		[^{3}H]Glu incorporated, pmol (%)	
L-AMT, μM	DL-γ,γ-F_2MTX, μM	1 h	2 h
40	0	477 (100)	959 (100)
40	200	343 (72)	639 (67)
0	200	2 (<1)	0 (0)

[a] CCRF-CEM FPGS was incubated with 0.5 mM L-[^{3}H]Glu (8.2 dpm/pmol) and L-AMT in the presence or absence of DL-γ,γ-F_2MTX for the indicated time under otherwise standard conditions and [^{3}H]Glu incorporation was quantitated (single data points) as described in the Experimental Section.

Table 4. Inhibition by Antifolates of Purified Human Dihydrofolate Reductase (DHFR) and Uptake of [^{3}H]MTX[a]

	IC_{50}	
antifolate	DHFR, μM	[^{3}H]MTX uptake, μM
AMT	nd	3.0 ± 0.3
L-MTX	0.72 ± 0.04	14.5 ± 1.5
DL-γ,γ-F_2MTX	1.53 ± 0.03	8.72 ± 0.5

[a] Uptake experiments employed human leukemia (CCRF-CEM) cells. nd = not determined.

FMTX, and β,β-F_2MTX.[5,6,30] FPGS substrate activities of these compounds are significantly different depending on the position or number of fluorine atoms at the glutamyl portion (β,β-F_2MTX > MTX ≫ *erythro*-γ-FMTX > *threo*-γ-FMTX ≈ DL-γ,γ-F_2MTX). The latter two compounds display very poor substrate activity near the limit of detection of the FPGS assay. However, it appears from comparison of previous data[5] with those presented here that DL-γ,γ-F_2MTX is probably the poorer substrate. All of the analogues, however, retain other biochemical properties (e.g., DHFR inhibitory activity) almost identical to those of MTX. Regardless of their detailed mechanism of binding to FPGS, availability of these fluorinated analogues of MTX is of great importance for studying the role of polyglutamylation in antifolate cytotoxicity.

Experimental Section

Melting points were obtained on a Thomas-Hoover Mel-Temp apparatus and are uncorrected. 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 NMR and ^{13}C NMR spectra were recorded on either Bruker AM-300 or AM-360 spectrometers. ^{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, University of Michigan. UV spectra were obtained on a Beckman Model DU-7 spectrophotometer. Radioactivity was measured with a Packard liquid scintillation analyzer, Model 1600 TR.

Table 5. Growth Inhibition of Parental CCRF-CEM and Its Methotrexate-Resistant Sublines during Continuous (120 h) Exposure to L-MTX or DL-γ,γ-F_2MTX[a]

	ED_{50} (nM)			
drug	CCRF-CEM	R1 (↑ DHFR)[b]	R2 (↓ transport)[c]	R30dm (↓ Glu_n)[d]
L-MTX	13.7 ± 0.5 (n = 6)	655 ± 25 (n = 2)	2550 ± 150 (n = 2)	15.5 ± 0.5 (n = 2)
DL-γ,γ-F_2MTX	29.0 ± 3.4 (n = 6)	700 ± 20 (n = 2)	2150 ± 350 (n = 2)	24.3 ± 4.8 (n = 2)

[a] Average values are presented ± range for n = 2 and ± SD for n = 6. [b] CCRF-CEM subline resistant to MTX solely as a result of a 25-fold increase in wild-type DHFR protein and activity.[24] [c] CCRF-CEM subline resistant to MTX solely as a result of decreased MTX influx (normal levels of parental DHFR present).[25] [d] CCRF-CEM subline resistant to MTX solely as a result of decreased polyglutamylation; this cell line has 1% of the FPGS-specific activity (measured with MTX as the folate substrate) of parental CCRF-CEM.[26]

MTX was a generous gift of the National Cancer Institute and of Immunex (Seattle, WA). Aminopterin was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Glutamic acid was purchased from DuPont NEN Research Products (Boston, MA). [^{3}H]MTX was obtained from Moravek Biochemicals Inc. (Brea, CA). Concentrations of all solutions of MTX and fluoroglutamate-containing analogues, including DL-γ,γ-F_2-MTX, were determined using the extinction coefficient of MTX.[28] The synthetic methods for preparing L-*threo*-4-fluoroglutamic acid[29] and DL-3,3-difluoroglutamic acid[10,30] were recently reported.

***N,N*-Diethyl-4-carbethoxy-2,2-difluoro-3-hydroxy-4-nitrobutanamide (7).** Diethylamine (1.32 g, 18.0 mmol) was added to a solution of **6**[12] (2.63 g, 11.7 mmol) and ethyl nitroacetate (1.60 g, 11.7 mmol) in THF (15 mL) at 0 °C, and the mixture was stirred at ambient temperature for 5 h. The reaction mixture was cooled to 0 °C, diluted with ethyl acetate (30 mL), and poured into 0.5 N HCl (30 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (30 mL × 2). The combined extracts were dried over Na_2SO_4 and evaporated to give 3.98 g of a yellow oil **7** as a mixture (53:47) of diastereomers. This compound was used without further purification: R_f 0.21 (hexane−EtOAc, 4:1); ^{1}H NMR ($CDCl_3$) δ 1.1−1.4 (m, 9 H), 3.3−3.6 (m, 4 H), 5.0−5.3 (m, 2 H), 5.52 (d, J = 6.6 Hz, 1 H) and 5.58 (d, J = 7.6 Hz, 1 H); ^{19}F NMR ($CDCl_3$) major isomer δ −37.5 (dd, J = 15.5, 300 Hz), −30.4 (dd, J = 6.2, 300 Hz), minor isomer δ −38.1 (dd, J = 13.9, 300 Hz), −31.0 (dd, J = 6.2, 300 Hz).

***N,N*-Diethyl-3-acetoxy-4-carbethoxy-2,2-difluoro-4-nitrobutanamide (8).** The crude product **7** (3.98 g) was

dissolved in dry dichloromethane (15 mL). To the solution at 0 °C were added acetic anhydride (1.53 g, 15.0 mmol) and three drops of concentrated sulfuric acid, and the mixture was stirred at ambient temperature for 12 h. The reaction mixture was diluted with dichloromethane (30 mL) and poured into H_2O (40 mL). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (30 mL × 2). The combined extracts were dried over $MgSO_4$ and evaporated to dryness to give 4.38 g of a yellow oil **8** as a mixture (56:44) of diastereomers. This compound was used without further purification: R_f 0.36 (hexane−EtOAc, 4:1); ^{1}H NMR ($CDCl_3$) δ 1.1−1.4 (m, 9 H), 2.12 (s, 3 H), 3.2−3.5 (m, 4 H), 4.2−4.4 (m, 2 H), 5.75 (d, J = 4.4 Hz, 1 H) and 5.84 (d, J = 6.5 Hz, 1 H), 6.45 (dt, J = 16.1, 6.6 Hz, 1 H) and 6.60 (ddd, J = 4.4, 7.8, 17.1 Hz, 1 H); ^{19}F NMR ($CDCl_3$) major isomer δ −34.1 (dd, J = 17.0, 288 Hz), −30.0 (dd, J = 7.8, 288 Hz), minor isomer δ −34.4 (dd, J = 17.0, 288 Hz), −31.8 (dd, J = 7.8, 288 Hz).

***N,N*-Diethyl-4-carbethoxy-2,2-difluoro-4-nitrobutanamide (9).** The crude product **8** (750 mg) was dissolved in dry THF (15 mL). Sodium borohydride (75 mg, 2.0 mmol) was added to the solution at 0 °C, and the mixture was stirred at ambient temperature for 30 min. The resulting mixture was cooled to 0 °C, and then ethyl acetate (15 mL) and 0.5 N HCl (15 mL) were added. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (15 mL × 2). The combined extracts were dried over Na_2SO_4 and evaporated to dryness. The resulting oil was chromatographed on silica gel (hexane−EtOAc, 5:1) to give a colorless oil **9** (404 mg, 1.4 mmol) in 68% yield from **6**: R_f 0.30 (hexane−EtOAc, 4:1); IR (neat) 3000, 1760, 1660, 1570 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.17 (t, J = 7.1 Hz, 3 H), 1.20 (t, J = 7.1 Hz, 3 H), 1.32 (t, J = 7.2 Hz, 3 H), 3.0−3.4 (m, 2 H), 3.39 (q, J = 7.1 Hz, 2 H), 3.50 (tq, J = 1.5, 7.1 Hz, 2 H), 4.32 (q, J = 7.2 Hz, 1 H), 5.54 (dd, J = 4.1, 8.8 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 12.2, 13.8, 14.2, 35.7 (dd, J = 24.1, 25.6 Hz), 41.6, 41.8 (t, J = 6.1 Hz), 63.7, 82.8 (dd, J = 4.1, 4.3 Hz), 117.6 (t, J = 258 Hz), 161.1 (t, J = 28.0 Hz), 163.5; ^{19}F NMR ($CDCl_3$) δ −24.9 (ddd, J = 11.4, 18.3, 288 Hz), −22.6 (dt, J = 18.3, 288 Hz); MS (EI) *m/e* (rel intensity) 296 (M^+, 1.9), 281 (13.4), 250 (7.5), 175 (94.4), 100 (100); HRMS (EI) *m/e* calcd for $C_{11}H_{18}F_2N_2O_5$ (M^+) 296.1184, found 296.1192. Anal. ($C_{11}H_{18}F_2N_2O_5$) C, H, N.

DL-4,4-Difluoroglutamic Acid (1). Raney Ni[31] (wet volume of 1 mL) was added to a solution of **9** (453 mg, 1.5 mmol) in ethanol (5 mL), and the mixture was shaken under hydrogen (40 psi) at ambient temperature for 12 h. The catalyst was removed by filtration and washed with ethyl acetate. The combined filtrate and washings were evaporated to dryness and dissolved in 12 N HCl (10 mL). The mixture was heated at 100 °C for 12 h and evaporated to dryness. The resulting yellow solid was dissolved in H_2O (3 mL) and chromatographed on anion-exchange resin (AG1 X8, BioRad, 20 mL wet volume) with H_2O and then 4 N AcOH as the eluants. Fractions containing the product were lyophilized, triturated with dry ether, and filtered to give 152 mg (54% yield) of the product **1** as a white solid. The sample for elemental analysis and bioassay was prepared by recrystallization from H_2O: R_f 0.78 (EtOH−H_2O, 7:3); mp 175−7 °C dec; IR (KBr) 3424, 3143, 1708 cm^{-1}; ^{1}H NMR (D_2O) δ 2.71 (dddd, J = 8.5, 13.1, 20.2 Hz, 1 H), 2.89 (ddt, J = 13.8, 21.2, 15.5 Hz, 1 H), 4.38 (dd, J = 3.7, 8.5 Hz, 1 H); ^{13}C NMR (D_2O) δ 34.9 (t, J = 26.4 Hz), 48.8, 116.3 (t, J = 251 Hz), 168.3, 170.9; ^{19}F NMR (D_2O) δ −29.6 (dd, J = 15.1, 21.2 Hz), −28.4 (dd, J = 12.2, 21.4 Hz); MS (CI/NH_3) *m/e* (rel intensity) 183 (MNH_4^+ − H_2O, 100), 165 (9), 147 (13), 136 (21), 119 (32), 101 (36), 84 (9). Anal. ($C_5H_7F_2NO_4$) C, H, N.

DL-4,4-Difluoroglutamic Acid Di-*tert*-butyl Ester (10). A suspension of **1** (63 mg, 0.34 mmol) in dry dichloromethane (1.5 mL) was placed in a pressure bottle. The bottle was cooled to −30 °C, and concentrated H_2SO_4 (30 μL) was added. Isobutylene (1.5 mL) was then condensed within the bottle at the same temperature. The bottle was closed, and the mixture was stirred at ambient temperature for 5 days as the reaction mixture became homogeneous. The reaction solution was cooled to −30 °C, diluted with ethyl acetate (10 mL), and poured into 20% aqueous K_2CO_3 solution (10 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (10 mL × 2). The combined extracts were dried over $MgSO_4$ and evaporated. The resulting oil was purified by silica gel column chromatography (hexane−EtOAc = 4:1) to give 80 mg (80% yield) of the product **10** as a yellow solid: mp 43−45 °C; R_f 0.60 (hexane−EtOAc = 1:1); IR (neat) 3396, 2979, 1763, 1748 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9 H), 1.51 (s, 9 H), 2.32 (dddd, J = 9.6, 12.5, 14.6, 20.2 Hz, 1 H), 2.50 (dq, J = 4.2, 14.6 Hz, 1 H), 3.61 (dd, J = 4.2, 9.6 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 27.4 (9 C), 27.7 (9 C), 38.8 (t, J = 24.0 Hz), 50.2 (dd, J = 3.7, 6.1 Hz), 81.8, 84.3, 115.4 (t, J = 249 Hz), 162.8 (t, J = 31.7 Hz), 173.3; ^{19}F NMR ($CDCl_3$) δ −31.7 (ddd, J = 13.8, 19.9, 261 Hz, 1 F), −26.6 (dt, J = 261, 15.3 Hz, 1 F); MS (CI/NH_3) *m/e* (rel intensity) 296 (MH^+, 100), 240 (98.0), 239 (61.3), 184 (21.2); HRMS (CI/NH_3) *m/e* calcd for $C_{13}H_{23}F_2NO_4H$ (MH^+) 296.1673, found 296.1684. Anal. ($C_{13}H_{23}F_2NO_4$) C, H, N.

DL-*N*-[4-[*N*′-(Benzyloxycarbonyl)-*N*′-methylamino]benzoyl]-4,4-difluoroglutamic Acid Di-*tert*-butyl Ester (11). To a solution of 4-[*N*-(benzyloxycarbonyl)-*N*-methylamino]-benzoic acid (80 mg, 0.27 mmol) in dry DMF (5 mL) were added DCC (105 mg, 0.42 mmol) and HOBt (84 mg, 0.60 mmol) at 0 °C, and the mixture was stirred at ambient temperature for 5 min. A solution of **10** (80 mg, 0.27 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 was removed under reduced pressure. The resulting crude oil was diluted with hexane−EtOAc (1:1) and again filtered. The filtrate was evaporated and the resulting oil was purified by silica gel chromatography (hexane−EtOAc = 4:1) to give 149 mg (98% yield) of the product **11** as a colorless oil: R_f 0.57 (hexane−EtOAc = 1:1); IR (neat) 3346, 2981, 2931, 1750, 1715, 1666 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9 H), 1.49 (s, 9 H), 2.6−2.9 (m, 2 H), 3.34 (s, 3 H), 4.85 (dt, J = 7.3, 5.6 Hz, 1 H), 5.17 (s, 2 H), 6.81 (d, J = 7.5 Hz, 1 H), 7.30−7.50 (m, 7 H), 7.80 (d, J = 8.7 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 27.9 (3 C), 28.1 (3 C), 36.0 (t, J = 22.9 Hz), 37.6, 49.0, 67.9, 83.4, 85.3, 115.3 (t, J = 253 Hz), 125.2 (2 C), 127.9 (2 C), 128.0 (2 C), 128.2, 128.7 (2 C), 131.0, 136.4, 146.4, 155.2, 162.6 (t, J = 31.7 Hz), 166.2, 169.6; ^{19}F NMR ($CDCl_3$) δ −28.6 (dt, J = 265, 16.4 Hz, 1 F), −26.9 (ddd, J = 12.4, 17.8, 265 Hz, 1 F); MS (EI) *m/e* (rel intensity) 562 (M^+, 19.3), 489 (2.7), 433 (17.9), 405 (35.5), 268 (61.6), 91 (100); HRMS (EI) *m/e* calcd for $C_{29}H_{36}F_2N_2O_7$ (M^+) 562.2490, found 562.2501. Anal. ($C_{29}H_{36}F_2N_2O_7$) C, H, N.

DL-*N*-[4-(Methylamino)benzoyl]-4,4-difluoroglutamic Acid Di-*tert*-butyl Ester (12). Palladium hydroxide on carbon (10 mg) was added to a solution of **11** (124 mg, 0.22 mmol) in dry ethanol (4 mL), and the mixture was shaken under hydrogen (30 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 pure **12** (64 mg, 0.15 mmol) in 69% yield: R_f 0.50 (hexane−EtOAc = 1:1); IR (neat) 3423, 3360, 2973, 2938, 1750, 1645; ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9 H), 1.47 (s, 9 H), 2.50−2.90 (m, 2 H), 2.83 (s, 3 H), 4.05−4.50 (br, 1 H), 4.84 (dt, J = 7.2, 5.6 Hz, 1 H), 6.53 (d, J = 8.3 Hz, 2 H), 6.68 (d, J = 7.4 Hz, 1 H), 7.62 (d, J = 8.3 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 27.9 (3 C), 28.1 (3 C), 30.4, 36.2 (t, J = 22.9 Hz), 48.7, 83.1, 85.1, 111.6 (2 C), 115.4 (t, J = 252 Hz), 121.9, 129.1 (2 C), 152.4, 166.0 (t, J = 32.6), 167.0, 170.2; ^{19}F NMR ($CDCl_3$) δ −28.6 (dt, J = 265, 17.2 Hz), −26.5 (ddd, J = 265, 13.6, 17.0 Hz); MS (EI) *m/e* (rel intensity) 428 (M^+, 10.3), 299 (9.8), 271 (9.6), 149 (8.8), 134 (100); HRMS (EI) *m/e* calcd for $C_{21}H_{30}F_2N_2O_5$ (M^+) 428.2123, found 428.2141. Anal. ($C_{21}H_{30}F_2N_2O_5$) C, H, N.

DL-γ,γ-Difluoromethotrexate (2). 6-(Bromomethyl)-2,4-pteridinediamine hydrobromide[15] (101 mg, 0.30 mmol) was added to a solution of **12** (64 mg, 0.15 mmol) in dry DMAC (1.5 mL) at 0 °C, and the mixture was stirred at 55 °C for 6 h. After additional stirring (18 h) at ambient temperature, the mixture was evaporated to dryness and dissolved in trifluoroacetic acid (5 mL). The mixture was stirred at ambient temperature for 40 min and evaporated to dryness. The crude product was purified by DEAE-cellulose chromatography (DE53, Whatman) [0.1−0.9 M triethylammonium carbonate

(TEAB) buffer (pH = 7.5) gradient] to afford 60 mg (61% yield based on absorption spectra) of γ,γ-difluoromethotrexate **2** as a triethylammonium salt: ^{1}H NMR (D_2O) δ 2.5−2.7 (m, 2 H), 2.95 (s, 3 H), 4.47 (s, 2 H), 4.51 (dd, J = 4.1, 8.7 Hz, 1 H), 6.55 (d, J = 8.7 Hz, 2 H), 7.49 (d, J = 8.7 Hz, 2 H), 8.34 (s, 1 H); ^{13}C NMR (D_2O) δ 36.6 (t, J = 23.6 Hz), 38.9, 50.6, 55.0, 111.9 (2 C), 117.6 (t, J = 250 Hz), 120.8, 122.3, 129.1, 148.6, 149.4, 151.8, 153.0, 161.8, 163.0, 169.2, 170.5, 178.0; ^{19}F NMR (D_2O) δ −28.5 (ddd, J = 14.4, 19.9, 244 Hz), −27.7 (ddd, J = 13.5, 18.5, 244 Hz); UV λ_{max} (pH = 13) 258, 302, 371; MS (FAB^+) m/e (rel intensity) 491 (MH^+, 10.5), 308 (6.1), 175 (10.2), 118 (30.4), 102 (100); HRMS (FAB^+) m/e calcd for $C_{20}H_{20}F_2N_8O_5H$ (MH^+) 491.1603, found 491.1607. Reversed-phase HPLC[17] t_R = 13.2 min; anion-exchange HPLC[18] t_R = 12.2 min.

FPGS Assays. CCRF-CEM FPGS was partially purified as previously described.[27,32] Unless otherwise indicated, FPGS was assayed as previously described.[16,18] Standard assay mixtures (250 μL) which contained 100 mM Tris-HCl, pH 8.85 (at room temperature), 10 mM $MgCl_2$, 5 mM ATP, 100 mM BME, 20 mM KCl, glutamate or fluorinated glutamates, and appropriate pteroyl substrates were initiated by addition of CCRF-CEM FPGS (1170 units) and incubated at 37 °C for the indicated time. After the addition of ice-cold 5 mM L-Glu (1 mL, pH = 7.5) containing 25 mM 2-mercaptoethanol, the assay mixtures were loaded onto DEAE-cellulose (DE52, Whatman) minicolumns equilibrated with 10 mM Tris-HCl, pH 7.5, containing 110 mM NaCl (conductivity = 11.5 mS/cm). The columns were washed with the same buffer to separate unreacted [^{3}H]glutamic acid from that incorporated into pteroyl substrates. Minicolumns were then eluted with 0.1 N HCl (3 mL) to obtain polyglutamate products. For HPLC analysis, 50 mM phosphoric acid was used for elution instead of 0.1 N HCl. One unit of FPGS catalyzes incorporation of 1 pmol of [^{3}H]Glu/h.

HPLC Analysis of FPGS Reaction Products.[16−18] The 50 mM phosphoric acid elution of the FPGS assay columns was filtered, and the filtrate injected into a C_{18} reversed-phase column (0.46 × 25 cm, Vydac 218TP54) previously equilibrated with 0.1 M sodium acetate (pH = 5.5) containing 4% acetonitrile. The column was washed isocratically with this buffer at 1 mL/min for 10 min and then with a linear gradient from 4 to 12.4% acetonitrile. Fractions were collected at a rate of 1 min/fraction, and radioactivity in each fraction was quantitated by liquid scintillation counting.

DHFR Inhibition. CCRF-CEM DHFR was partially purified and assayed at 37 °C as described previously.[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 antifolate to standard DHFR assays and measuring the remaining activity. The IC_{50} values for DHFR inhibition were determined graphically from plots of net activity versus antifolate concentration.

Cell Growth Inhibition. Inhibition of the growth of CCRF-CEM cells and its MTX-resistant sublines in continuous (120 h) drug exposure was measured as previously described.[26] EC_{50} values were determined graphically from plots of percentage growth relative to untreated control growth versus the logarithm of drug concentration.

Acknowledgment. This work was supported by research grants CA28097 (J.K.C.), CA13038 (J.J.M.), and CA16056 (J.J.M.) from the National Cancer Institute. We gratefully acknowledge the technical assistance of Holly and Robert Bogaudo, Wanda Bolanowska, Jill Galvin, and William Haile.

References

(1) McGuire, J. J.; Coward, J. K. In *Folates and Pterins*; Blakley, R. L., Benkovic, S. J., Eds.; John Wiley & Sons: New York, 1984; Vol. 1, pp 135−190.
(2) Shane, B. Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vitam. Horm.* **1989**, *45*, 263−335.
(3) McGuire, J. J.; Coward, J. K. DL-*threo*-4-Fluoroglutamic acid: A chain terminating inhibitor of folylpolyglutamate synthetase. *J. Biol. Chem.* **1985**, *260*, 6747−6754.
(4) McGuire, J. J.; Haile, W. H.; Bey, P.; Coward, J. K. DL-3,3-Difluoroglutamate: An enhancer of folylpolyglutamate elongation. *J. Biol. Chem.* **1990**, *265*, 14073−14079.
(5) McGuire, J. J.; Graber, M.; Licato, N.; Vincenz, C.; Coward, J. K.; Nimec, Z.; Galivan, J. Biochemical and growth inhibitory effects of the *erythro* and *threo* isomers of γ-fluoromethotrexate, a methotrexate analogue defective in polyglutamylation. *Cancer Res.* **1989**, *49*, 4517−4525.
(6) McGuire, J. J.; Hart, B. P.; Haile, W. H.; Rhee, M.; Galivan, J.; Coward, J. K. DL-β,β-Difluoroglutamic acid mediates position-dependent enhancement or termination of pteroylpoly (γ-glutamate) synthesis catalyzed by folylpolyglutamate synthetase. *Arch. Biochem. Biophys.* **1995**, *321*, 319−328.
(7) Coward, J. K.; McGuire, J. J.; Galivan, J. In *Selective Fluorination in Organic and Bioorganic Chemistry*; Welch, J. T., Ed.; American Chemical Society: Washington, D.C., 1991; pp 196−204.
(8) Kitagawa, O.; Hashimoto, A.; Kobayashi, Y.; Taguchi, T. Michael addition of 2,2-difluoroketene silyl acetal. Preparation of 4,4-difluoroglutamic acid and 5,5-difluorolysine. *Chem. Lett.* **1990**, 1307−1310.
(9) Yang, Z.-Y.; Burton, D. J. A new approach to α,α-difluoro-functionalized esters. *J. Org. Chem.* **1991**, *56*, 5125−5132.
(10) Hart, B. P.; Coward, J. K. The synthesis of DL-3,3-difluoroglutamic acid from a 3-oxoprolinol derivative. *Tetrahedron Lett.* **1993**, *34*, 4917−4920.
(11) Sufrin, J. R.; Balasubramanian, T. M.; Vora, C. M.; Marshall, G. R. Synthetic approaches to peptide analogues containing 4,4-difluoro-L-proline and 4-keto-L-proline. *Int. J. Pept. Protein Res.* **1982**, *20*, 438−442.
(12) Tsukamoto, T.; Kitazume, T. Preparation and reaction of difluorinated malonaldehydic acid derivatives: A new route to functionalized α,α-difluorinated esters and amides. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1177−1181.
(13) Martynov, V. F.; Grebenkina, V. M. Fluorine-containing carbonyl compounds in the knoevenagel reaction. *J. Gen. Chem. USSR* **1968**, *38*, 999−1001.
(14) Wollenberg, R. H.; Miller, S. J. Nitroalkane synthesis. A convenient method for aldehyde reductive nitromethylation. *Tetrahedron Lett.* **1978**, *35*, 3219−3222.
(15) Piper, J. R.; Montgomery, J. A. Preparation of 6-(bromomethyl)-2,4-pteridinediamine hydrobromide and its use in improved syntheses of methotrexate and related compounds. *J. Org. Chem.* **1977**, *42*, 208−211.
(16) McGuire, J. J.; Hsieh, P.; Coward, J. K.; Bertino, J. R. Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. *J. Biol. Chem.* **1980**, *255*, 5776−5788.
(17) Nimec, Z.; Galivan, J. Regulatory aspects of the glutamylation of methotrexate in cultured hepatoma cells. *Arch. Biochem. Biophys.* **1983**, *226*, 671−680.
(18) McGuire, J. J.; Hsieh, P.; Coward, J. K.; Bertino, J. R. *In vitro* methotrexate polyglutamate synthesis by rat liver folylpolyglutamate synthetase and inhibition by bromosulfophthalein. *Adv. Exp. Med. Biol.* **1983**, *163*, 199−214.
(19) Cichowicz, D. J.; Cook, J. D.; George, S.; Shane, B. In *Proceedings of the Second Workshop on Folyl and Antifolylpolyglutamates*; Goldman, I. D., Ed.; Praeger: New York, 1985; pp 7−13.
(20) George, S.; Cichowicz, D. J.; Shane, B. Mammalian folylpoly-γ-glutamate synthetase. 3. Specificity for folate analogues. *Biochemistry* **1987**, *26*, 522−529.
(21) McGuire, J. J.; Bolanowska, W. E.; Piper, J. R. Structural specificity of inhibition of human folylpolyglutamate synthetase by ornithine-containing folate analogs. *Biochem. Pharmacol.* **1988**, *37*, 3931−3939.
(22) Cramer, S. M.; Schornagel, J. H.; Kalghatgi, K. K.; Bertino, J. R.; Horvath, C. Occurrence and significance of D-methotrexate as a contaminant of commercial methotrexate. *Cancer Res.* **1984**, *44*, 1843−1846.
(23) McGuire, J. J.; Bolanowska, W. E.; Coward, J. K.; Sherwood, R. F.; Russell, C. A.; Felschow, D. M. Biochemical and biological properties of methotrexate analogs containing D-glutamic acid or D-*erythro*,*threo*-4-fluoroglutamic acid. *Biochem. Pharmacol.* **1991**, *42*, 2400−2403.
(24) Mini, E.; Srimatkandada, S.; Medina, W. D.; Moroson, B. A.; Carman, M. D.; Bertino, J. R. Molecular and karyological analysis of methotrexate-resistant and -sensitive human leukemic CCRF-CEM cells. *Cancer Res.* **1985**, *45*, 317−325.
(25) Rosowsky, A.; Lazaraus, H.; Yuan, G. C.; Beltz, W. R.; Mangini, L.; Abelson, H. T.; Modest, E. J.; Frei, E. I. Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts. *Biochem. Pharmacol.* **1980**, *29*, 648−652.
(26) McCloskey, D. E.; McGuire, J. J.; Russell, C. A.; Rowan, B. G.; Bertino, J. R.; Pizzorno, G.; Mini, E. Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. *J. Biol. Chem.* **1991**, *266*, 6181−6187.

(27) McGuire, J. J.; Russell, C. A.; Bolanowska, W. E.; Freitag, C. M.; Jones, C. S.; Kalman, T. I. Biochemical and growth inhibition studies of methotrexate and aminopterin analogues containing a tetrazole ring in place of the γ-carboxyl group. *Cancer Res.* **1990**, *50*, 1726−1731.
(28) Temple, J., C.; Montgomery, J. A. In *Folates and Pterins*; Blakley, R. L., Benkovic, S. J., Eds.; John Wiley & Sons: New York, 1984; Vol. 1, pp 61−120.
(29) Hudlicky, M. Stereospecific syntheses of all four stereoisomers of 4-fluoroglutamic acid. *J. Fluorine Chem.* **1993**, *60*, 193−210.
(30) Hart, B. P.; Licato, N. J.; Bolanowska, W. E.; McGuire, J. J.; Coward, J. K. The synthesis and biological activity of folic acid and methotrexate analogues containing L-*threo*(2*S*,4*S*)4-fluoroglutamic acid and DL-3,3-difluoroglutamic acid. *J. Med. Chem.* **1996**, *39*, 56−65.
(31) Mozingo, R. Catalyst, Raney Nickel, W-2. *Organic Syntheses*; Wiley: New York, 1955, Collect. Vol. III, pp 181−183.
(32) McGuire, J. J.; Bergoltz, V. V.; Heitzman, K. J.; Haile, W. H.; Russell, C. A.; Bolanowska, W. E.; Kotake, Y.; Haneda, T.; Nomura, H. Novel 6,5-fused-ring heterocyclic antifolates: Biochemical and biological characterization. *Cancer Res.* **1994**, *54*, 2673−2679.

JM950514M