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

Barry P. Hart,[†] William H. Haile,[‡] Nicholas J. Licato,[†] Wanda E. Bolanowska,[‡] John J. McGuire,[‡] and James K. Coward*[,†]

Departments of Chemistry and Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055, and Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, New York 14263

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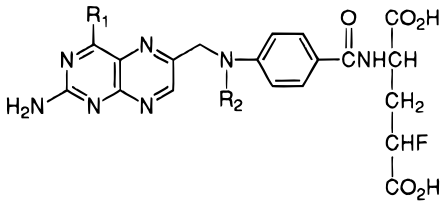
The stereospecific syntheses of L-*threo*-γ-fluoromethotrexate (**1*t***) and L-*threo*-γ-fluorofolic acid (**3*t***) are reported. Compounds **1*t*** and **3*t*** have no substrate activity with folylpoly-γ-glutamate synthetase isolated from CCRF-CEM human leukemia cells, and compound **1*t*** inhibits human dihydrofolate reductase at similar levels as methotrexate. The synthesis of DL-3,3-difluoroglutamic acid (**6**) and its incorporation into DL-β,β-difluorofolic acid (**4**) are also reported. Compound **4** acts as a better substrate for human CCRF-CEM folylpoly-γ-glutamate synthetase than folic acid (V/K = ca. 7-fold greater). Thus, replacement of the glutamate moiety of methotrexate and folic acid with 4-fluoroglutamic acid and 3,3-difluoroglutamic acid results in folates and antifolates with altered polyglutamylation activity.

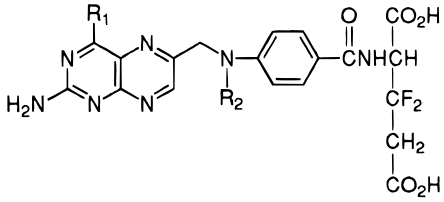
Introduction

The biosynthesis of poly-γ-glutamate "conjugates" is an important process in one-carbon biochemistry involving folate-dependent enzymes and in the cytotoxicity mediated by a variety of folate analogues such as methotrexate (MTX).[1,2] Previous publications from our laboratories have documented the use of fluorinated glutamic acids[3,4] and the corresponding fluoroglutamate-containing analogues of MTX[5,6] as informative probes to investigate the polyglutamylation process. Specifically, we have reported on the biological activity of γ-fluoromethotrexate (γ-FMTX, **1**)[5,7,8] and, more recently, β,β-difluoromethotrexate (β,β-F_2MTX, **2**).[6,9] The well-documented effects of the reduced folate, 5-formyltetrahydrofolic acid (leucovorin) in the rescue of cells treated with "high-dose" MTX chemotherapy[10] and the potentiation of 5-fluorouracil cytotoxicity[11] suggests that fluoroglutamate-containing analogues of folic acid and leucovorin might serve as useful probes of the role of polyglutamylation in folic acid and/or leucovorin biochemistry and pharmacology in intact mammalian cells. With such cellular studies in mind, we have initiated a synthetic program to obtain folic acid (FA) analogues containing various fluoroglutamic acids (e.g., **3**, **4**) to complement our ongoing research on fluoroglutamate-containing analogues of MTX.

We have previously used racemic materials in our research because of the paucity of stereospecific syntheses of the requisite amino acids, e.g., **5** and **6**. Thus, 4-fluoroglutamic acid, **5**, as a mixture of all four possible isomers, is available via addition of the carbanion of diethyl fluoromalonate to methylacetamido acrylate.[5,12] This stereochemical heterogeneity of the precursor amino acids leads to stereochemical heterogeneity in the fluoroglutamate-containing drugs of interest in our research (Table 1). Although in the case of MTX it has been demonstrated that the D-isomer is biologically inactive,[13,14] it is not always a simple task to sort out

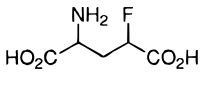


1 (γ-FMTX):R_1 = NH_2, R_2 = CH_3
3 (γ-FFA):R_1 = OH, R_2 = H

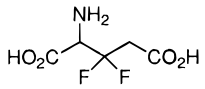


2 (β,β-F_2MTX):R_1 = NH_2, R_2 = CH_3
4 (β,β-F_2FA):R_1 = OH, R_2 = H

the complex biological results when using mixtures of stereoisomers. Therefore, we have undertaken the



5 (4-FGlu)



6 (3,3-F_2Glu)

stereospecific synthesis of fluorinated glutamic acids for use in the synthesis of several folate and MTX analogues in order to investigate the role of polyglutamylation in the biochemistry and pharmacology of the parent glutamate-containing compounds. Our initial research with racemic 4-fluoroglutamic acid, **5*et*** *(rac)*, demonstrated that the L-*threo* (2*S*,4*S*) isomer is preferentially used as a folylpoly-γ-glutamate synthetase (FPGS, EC 6.3.2.17) chain-terminating substrate.[3] On the basis of these observations, our initial synthetic target was L-*threo*-4-fluoroglutamic acid, **5*t***,[15] and in this paper we report on the synthesis of L-*threo*-γ-

[†] University of Michigan.
[‡] Roswell Park Cancer Institute.
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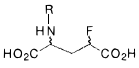
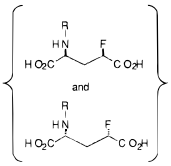
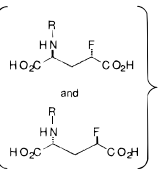
Table 1. Definition of Stereochemical Parameters: 4-FGlu (**5**), γ-FMTX (**1**), and γ-FFA (**3**)

Abbreviation[a]	Structure	Stereochemical Designation	Absolute Stereochemistry
et *(rac)*		DL-*erythro*, *threo*	2*RS*, 4*RS*
e *(rac)*		DL-*erythro*	2*S*, 4*R* and 2*R*, 4*S*
t *(rac)*		DL-*threo*	2*S*, 4*S* and 2*R*, 4*R*

[a] Throughout the text, including most notably the Experimental Section and Scheme 1, a number preceding the abbreviation indicates that the stereochemistry of the numbered compound is as indicated in this Table; e.g., **5*t*** is L-*threo*-4-fluoroglutamic acid where the absolute stereochemistry is 2*S*,4*S*. The use of the abbreviation *rac* indicates that the numbered compound is a racemic mixture; e.g., **5*t*** (*rac*) is DL-*threo*-4-fluoroglutamic acid with absolute stereochemistry of 2*S*,4*S*; 2*R*,4*R*. In the glutamyl portions of MTX or folate derivatives, the numbers 2 and 4 are replaced by α and γ, respectively, in order to distinguish the positions on the appended glutamate from the positions on the pteridine or *p*-aminobenzoyl moieties.

fluoromethotrexate (L-*t*-γ-FMTX, **1*t***) containing only the L-*threo* (2*S*,4*S*) diastereomer. In addition, we report the synthesis of L-*threo*-γ-fluorofolic acid (L-*t*-γ-FFA, **3*t***) containing the same stereochemically pure amino acid. As expected, the 4-fluoroglutamate-containing analogues, **1*t*** and **3*t***, are extremely poor substrates for FPGS.

Racemic 3,3-difluoroglutamic acid **6** can be obtained by a multistep synthesis from a blocked 3-oxoprolinol.[16] On the basis of our recent report of the synthesis and biochemical properties of DL-3,3-difluoroglutamate-containing MTX analogue, β,β-difluoromethotrexate (β,β-F_2MTX, **2**),[6,9] we have also synthesized the corresponding folic acid analogue, **4**. Consistent with observations on the β,β-F_2MTX analogue, **2**, the corresponding folate, β,β-difluorofolic acid (β,β-F_2FA, **4**), is an excellent FPGS substrate with V/K = ca. 7-fold greater than that of folic acid. Thus, the initial biochemical results support the hypothesis[17] that control of intracellular polyglutamylation of both folic acid and MTX can be effected by the judicious use of regiospecifically fluorinated glutamates incorporated into analogues of the parent vitamin or drug.

Chemistry

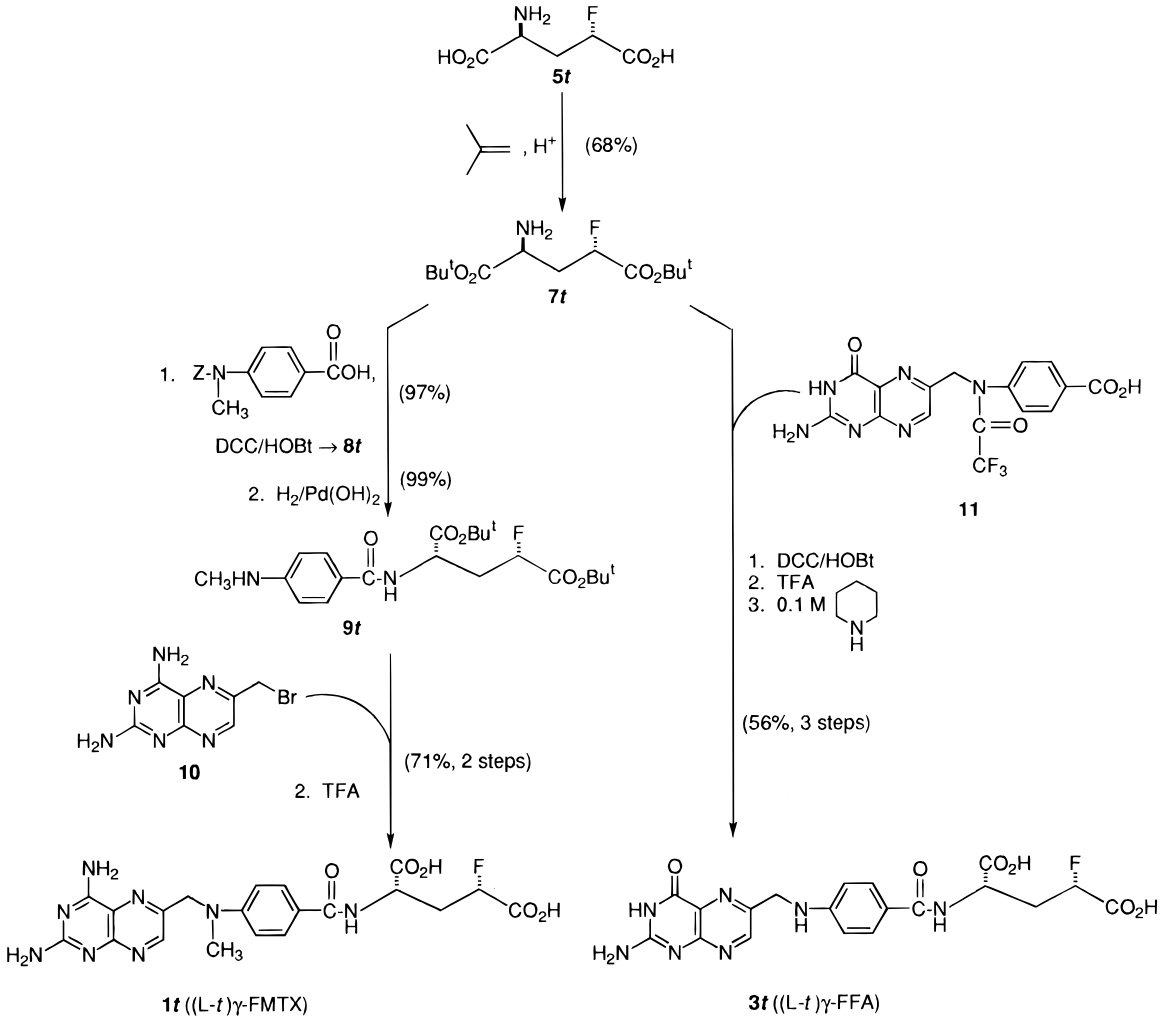
The synthesis of MTX analogues with various replacements for the glutamate moiety has been investigated extensively.[18] Recently, several fluorinated analogues of glutamic acid, namely the four possible stereoisomers of 4-fluoroglutamic acid, **5**,[19] and DL-3,3-difluoroglutamic acid, **6**,[16] have been prepared via appropriate fluorinated proline derivatives as synthetic intermediates. In the present work, the synthesis of fluorinated glutamic acid-containing analogues of MTX and folic acid is reported. These compounds were obtained through either C^9-N^{10} bond formation or pteroyl amide bond formation. The amide bond formation strategy is direct but does not allow for the facile incorporation of glutamic acid analogues into other modified pteridine ring systems. In previous work from this laboratory, iBCF- and DEPC-mediated coupling reactions between 2,4-diamino-N^{10}-methylpteroic acid and suitably protected γ-(fluoro)-glutamyl peptides resulted in only low to moderate yields of coupled product.[20,21] The synthesis of L-*t*-γ-FMTX (**1*t***) was achieved by the coupling of 2,4-diamino-6-(bromomethyl)pteridine (**10**) with **9*t***. In contrast, the fluorinated folates (**3*t*** and **4**) were obtained by the coupling of N^{10}-Tfa-pteroic acid (**11**), readily available from folic acid by enzyme-catalyzed hydrolysis,[22] with the corresponding fluoroglutamate di-*tert*-butyl ester (**7*t*** and **18**).

Synthesis of 4-Fluoroglutamate-Containing Analogues of MTX and Folic Acid. Initially, the synthesis of DL-4(*RS*)-fluoroglutamic acid, **5*et*** (*rac*), was achieved by the method of Buchanan,[12] and the diastereomers of **7*et*** (*rac*) were separated by silica gel column chromatography,[5] thereby providing **7*e*** (*rac*) and **7*t*** (*rac*). Analogues of MTX containing the two racemic diastereomers of **5**, **5*e*** (*rac*), and **5*t*** (*rac*), were synthesized and studied extensively.[5,8,14] More recently, the method of Hudlicky[19,23] has been used to effect the stereospecific synthesis of L-4(*S*)-fluoroglutamic acid, **5*t***, from protected L-4(*R*)-hydroxyproline. In the present work, the synthesis of L-*t*-γ-FMTX was achieved in 50% overall yield from **5*t*** (Scheme 1). The coupling of **7*t***, obtained from **5*t*** in 68% yield, with *N*-Me,*N*-Cbz-PABA provided **8*t*** in 97% yield followed by hydrogenolysis to afford **9*t*** in near quantitative yield. The coupling of 6-(bromomethyl)pteridine **10** with **9*t*** followed by TFA-mediated deprotection and purification by DEAE-cellulose chromatography provided **1*t***.

Previous studies comparing the effects of DL-*t*-γ-FMTX (**1*t*** (*rac*)) and D-*t*-γ-FMTX led to the conclusion that the biological activity of DL-*t*-γ-FMTX resulted almost exclusively from the L-isomer; however direct evidence to support this conclusion was lacking.[14] Accordingly, L-*t*-γ-FMTX (**1*t***) has now been synthesized as described above (Scheme 1). In order to determine whether racemization occurred during synthesis, the isomeric composition of the amino acid moiety was determined by limit digestion with CPG2, an enzyme which is specific for hydrolysis of L-amino acids contained in folate or antifolate structures.[14,24] Hydrolysis by CPG2 (0.54 unit) of 10−100 nmol of L-MTX, as described in the Experimental Section, was complete and quantitative in <1 min. In a parallel experiment, L-*t*-γ-FMTX was hydrolyzed to 97.5% of completion at a concentration equivalent to that of L-MTX. In contrast, DL-MTX and DL-*t*-γ-FMTX were hydrolyzed to only 49.4% and 48%, respectively, the extent of L-MTX under identical conditions. These results indicate that little, if any, racemization occurred during synthesis and that the product obtained, **1*t***, is the L-isomer.

The synthesis of folate analogues, **3*et*** (*rac*), **3*e*** (*rac*), and **3*t*** (*rac*) was achieved in 31−42% yield by coupling **11**, activated by isobutyl chloroformate, with the appropriate di-*tert*-butyl ester (**7*et*** (*rac*), **7e** (*rac*), or **7t** (*rac*)). Attempts to improve the overall yield of this coupling and deprotection sequence involved the exami-

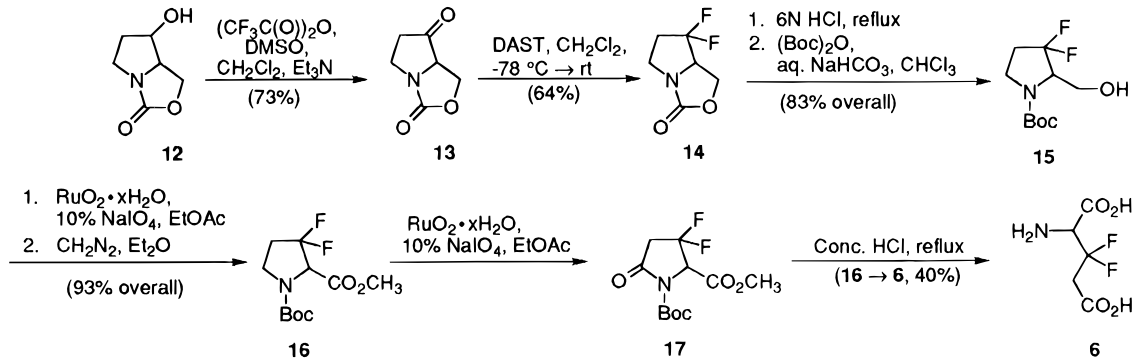
Scheme 1



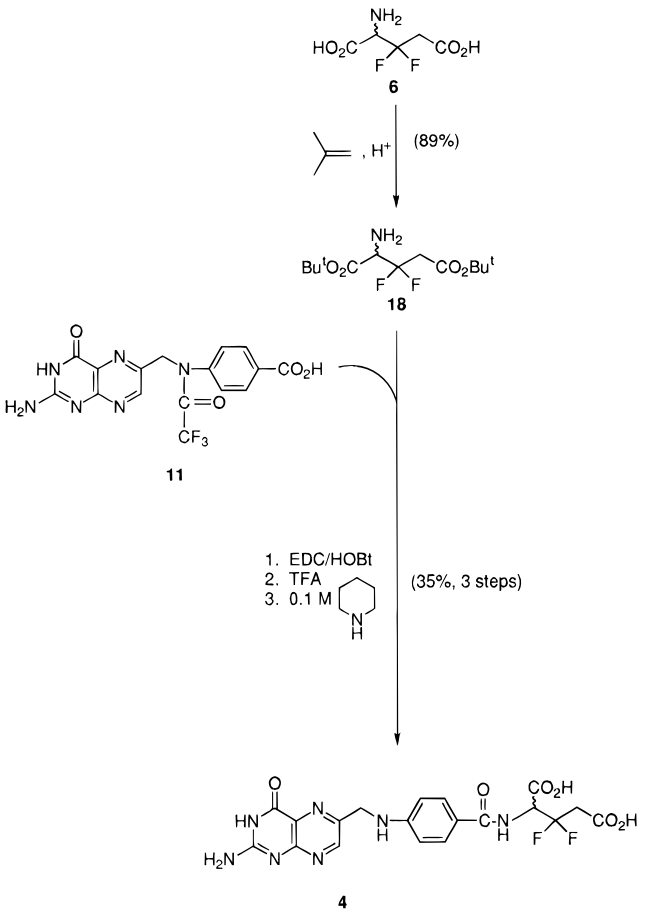
nation of several alternate coupling agents, e.g., iBCF, DEPC, BOP, DCC/HOBt. Ultimately, the DCC/HOBt-mediated coupling of **11** and **7*t***, derived from **5*t***, followed by deprotection provided **3*t*** in 56% overall yield (Scheme 1).

Synthesis of the 3,3-Difluoroglutamate-Containing Analogue of Folic Acid. DL-3,3-Difluoroglutamic acid **6** was synthesized from 6-hydroxy-1-aza-3-oxabicyclo[3.3.0]octan-2-one **(12)**, a bicyclic prolinol derivative (Scheme 2), as described briefly in a previous communication.[16] The oxidation of **12** under conditions reported by Swern[25] provided **13** in 73% yield. The (diethylamido)sulfur trifluoride (DAST)-mediated fluorination of ketone **13** to the geminal difluoromethylene compound **14** proceeded in 64% yield. Hydrolysis and Boc protection of **14** provided **15** in 83% yield. The alcohol **15** was readily converted to the methyl ester, **16**, by sequential oxidation to the acid and esterification. Compound **16** proved to be extremely resistant to RuO_4-mediated oxidation to lactam **17**. In contrast to the facile oxidation of appropriately protected proline derivatives[26] or a somewhat slower rate of oxidation of 4-fluoroproline derivatives,[19,27] the conversion of **16** to **17** required 288 h of stirring at room temperature. The reaction was monitored by NMR spectroscopy due to the instability of **17** on silica gel TLC. In the course of investigating the general feasibility of this oxidative process, several isomeric fluoroprolines were subjected

Scheme 2



Scheme 3



to the action of RuO_4 under a variety of conditions.[27] The overall effect of fluorine substitution is to retard the oxidation reaction. The magnitude of this decrease in rate is dependent on the location and extent of fluorine substitution relative to the site of oxidation.[27] DL-3,3-Difluoroglutamic acid **6** was obtained by the hydrolysis of crude **17** followed by anion-exchange chromatography and crystallization. The free acid **6** was converted to its di *tert*-butyl ester, **18,** by acid-catalyzed reaction with isobutylene. The EDC/HOBt-mediated coupling of **18** and **11** followed by TFA and piperidine deprotection provided **4** in 35% overall yield for three steps (Scheme 3).

Biochemistry

Substrate Activity of 4-Fluoroglutamate-Containing MTX and Folate Analogues with Folylpolyglutamate Synthetase. As expected from our earlier work with diastereomeric mixtures of γ-FMTX,[5] the stereochemically pure L-*threo* isomer ($\alpha S,\gamma S$) of γ-FMTX (**1*t***) is not a substrate for human FPGS (Figure 1) at concentrations up to 240 μM. With the folate analogues, the mixture of racemic *erythro, threo* diastereomers (**3*e,t*** (*rac*)) and the individual racemates (**3*e*** (*rac*), **3*t*** (*rac*)) were each compared to PteGlu as substrates for rat liver FPGS (Figure 2A). All analogues were extremely poor substrates relative to PteGlu. Similar results were obtained with human FPGS for the diastereomeric mixtures, **3*e,t*** (*rac*), **3*e*** (*rac*), and **3*t*** (*rac*) (data not shown) and for the stereochemically homogeneous analogue, **3*t*** (Figure 3). Since reduced folates are better FPGS substrates than the fully oxidized species,[1]

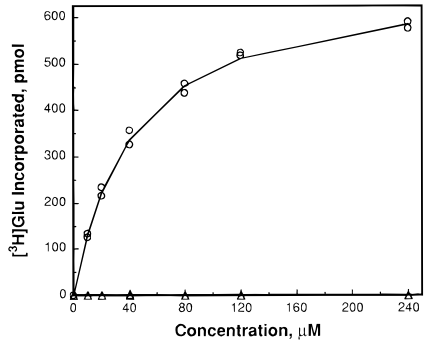


Figure 1. Substrate activity of MTX (○) and L-*t*-FMTX (**1*t***, △) with CCRF-CEM human leukemia cell FPGS.

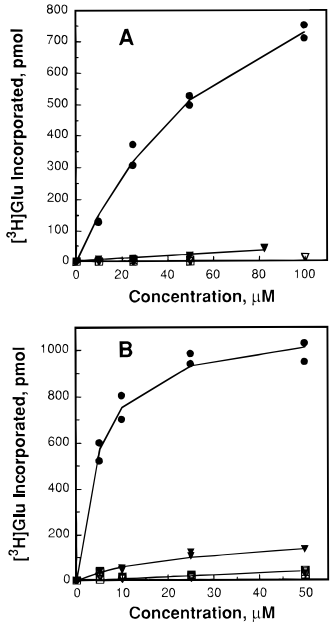


Figure 2. Substrate activity of indicated folates and 7,8-dihydrofolates with rat liver FPGS: (A) L-PteGlu (●), DL-*e,t*-PteFGlu (□), DL-*e*-PteFGlu (▼), and DL-*t*-PteFGlu (▽); (B) L-H_2PteGlu (●), DL-*e,t*-H_2PteFGlu (□), DL-*e*-H_2PteFGlu (▼), and DL-*t*-H_2PteFGlu (▽).

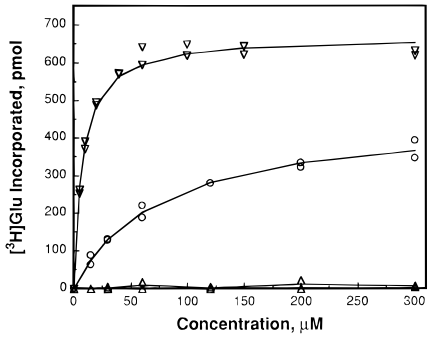


Figure 3. Substrate activity of L-PteGlu (○), L-*t*-PteFGlu (**3*t***, △), and DL-Pte(3,3-F_2Glu) (**4**, ▽) with CCRF-CEM human leukemia cell FPGS.

the 7,8-dihydro forms of the racemic mixed *erythro,threo* diastereomers and the individual racemates were also each tested for rat liver FPGS substrate activity and compared to H_2PteGlu (Figure 2B). H_2PteGlu is a much more active substrate (ca. 46-fold higher *V*/*K*) than PteGlu for rat liver FPGS. Slight activity of the dihydro forms of the analogues was noted; DL-*e*-H_2PteFGlu (*V*/*K* = 8.6 vs 232 for H_2PteGlu) was the most active of the two diastereomers, but it reached only 13% of the

maximal activity of the natural substrate. Similar low substrate activity of the dihydro derivatives was noted with human FPGS (data not shown).

The analogues were also tested for their ability to inhibit human CCRF-CEM FPGS activity using 30 μM aminopterin (AMT) as the substrate. Oxidized fluorofolates and the 7,8-dihydro forms are weak inhibitors of FPGS. Thus, the oxidized forms, **3*e,t*** (*rac*) and **3*t*** (*rac*) with $IC_{50} > 200$ μM and **3*e*** (*rac*) with $IC_{50} > 160$ μM), can be compared to the reduced forms, DL-*e,t*-H_2-PteFGlu (59% inhibition by 100 μM) and DL-*t*-H_2-PteFGlu (58% inhibition by 100 μM). Quantitative evaluation of the inhibition data is complicated by the weak substrate activity of the FGlu-containing analogues; it is clear, however, that the poor substrate activity reflects poor binding to the enzyme. These analogues do not bind tightly and also fail to participate in catalysis.

Substrate Activity of the 3,3-Difluoroglutamate-Containing Folate Analogue with Folylpolyglutamate Synthetase. The activity of β,β-F_2FA (**4**) as a substrate for human FPGS was studied, and **4** was found to be a better substrate than PteGlu (Figure 3). This increase in the catalytic efficiency for the L-isomer of β,β-F_2FA over PteGlu (V/K = 0.176 vs 0.024) is consistent with results comparing β,β-F_2MTX to MTX.[6] These data provide evidence that the replacement of the glutamate potion of folates and antifolates with 3,3-F_2-Glu will provide a "generic" means of increasing the polyglutamylation activity of these classes of compounds. However, this result is complicated by the fact that the FPGS-catalyzed polyglutamylation of β,β-F_2-FA and β,β-F_2MTX terminates after the addition of a single glutamate.[6] The resulting dipeptides are not substrates for FPGS. Further studies are underway to better understand the unusual FPGS activities of β,β-F_2FA and β,β-F_2MTX.

Activity of Reduced Forms of Fluorofolates as Substrates for Dihydrofolate Reductase. DL-*e,t*-FFA (**3*e,t*** (*rac*)), DL-*e*-FFA (**3*e*** (*rac*)), and DL-*t*-FFA (**3*t*** (*rac*)) were reduced to the 7,8-dihydro form, and each was tested in kinetic studies with CCRF-CEM human leukemia cell DHFR. Inhibition by MTX, a tight-binding inhibitor of DHFR,[28] was similar (50% inhibition achieved at 0.4 nM) whether H_2PteGlu or DL-*e,t*-H_2PteFGlu was the competing substrate, regardless of substrate concn in the range of 1−20 μM. The K_m for NADPH, the cosubstrate for DHFR, was 3.8 ± 0.5 μM ($n = 2$) or 3.0 ± 0.3 μM ($n = 2$) when determined in the presence of 3 μM H_2PteGlu or DL-*e,t*-H_2PteFGlu, respectively; thus the K_m for NADPH is unchanged. The standard spectrophotometric assay was not sensitive enough to determine exact K_m values for H_2PteGlu and DL-*e,t*-H_2PteFGlu, but both were <4 μM, while the apparent V_{max} values were identical. A second attempt using a more sensitive spectrophotometer and determining the K_m from a single complete reaction progress curve[29] led to the conclusion that the K_m values for H_2-PteGlu, DL-e,*t*-H_2PteFGlu, DL-*e*-H_2PteFGlu, and DL-*t*-H_2-PteFGlu were each <1 μM.

Substrate activity of 7,8-dihydro-DL-β,β-F_2FA was measured, and it was found that V_{max} of the reduced difluorofolate is ca. 0.74V_{max} of dihydrofolate. K_m values appear to be slightly lower than for dihydrofolate, suggesting that both the D- and L-isomers are substrates; this lack of stereospecificity may also be reflected in the lower value of V_{max} observed.

Table 2. Inhibition of Dihydrofolate Reductase Isolated from CCRF-CEM Human Leukemia Cells by Fluoroglutamate-Containing Analogues of Methotrexate[a]

compound	IC_{50}, nM experiment 1	IC_{50}, nM experiment 2
L-MTX	0.72 ± 0.07	0.60 ± 0.03
DL-MTX	1.18 ± 0.08	1.40 ± 0.06
L-*t*-γ-FMTX (**1*t***)	0.84 ± 0.10	
DL-*t*-γ-FMTX	1.35 ± 0.05	
DL-β,β-F_2MTX (**2**)		1.34 ± 0.03

[a] Average values are presented ± range ($n = 2$).

Activity of Fluorinated Analogues of MTX as Inhibitors of Dihydrofolate Reductase. Studies of the inhibition of isolated DHFR by L-MTX, DL-MTX, L-*t*-γ-FMTX, DL-*t*-γ-FMTX, and DL-β,β-F_2MTX are summarized by the data of Table 2. As expected, the racemic compounds are half as potent as the L-isomers as DHFR inhibitors, consistent with the conclusion that only the L-isomer of glutamic acid binds to DHFR.[13]

Conclusion

The stereospecific synthesis of (2*S*,4*S*)-4-fluoroglutamic acid and its incorporation into methotrexate and folic acid analogues results in (αS,γS)-γ-fluoromethotrexate, **1*t***, and (αS,γS)-γ-fluorofolic acid, **3*t***, for use in studying the role of poly-γ-glutamates in antifolate cytotoxicity and folate-dependent one-carbon biochemistry, respectively. Compound **1*t*** was found to be essentially inactive as a substrate for FPGS but is a potent inhibitor of DHFR with IC_{50} values comparable to those of L-MTX (Table 2).

The synthesis of **3** has been described previously in the literature,[30] but the reported synthesis led to a mixture of *erythro* and *threo* isomers in very low yield and no biological data were reported. The synthetic research described here affords *erythro*, **3*e*** (*rac*), and *threo*, **3*t*** (*rac*), diastereoisomers, separable by column chromatography, or the stereochemically pure L-*threo*-(2*S*,4*S*)-4-fluoroglutamate-containing analogue, **3*t***. Biochemical studies show that both oxidized and reduced fluorofolates are very poor substrates for FPGS. These results extend previous observations with racemic γ-fluoromethotrexate[5] that replacing the L-Glu portion of folates and antifolates such as methotrexate with (2*S*,4*S*)-4-FGlu will result in analogues of these compounds that are not converted to the poly-γ-glutamate conjugates. The stereochemically homogeneous compounds, **1*t*** and **3*t***, will facilitate ongoing research concerning the effect of polyglutamylation of folates and antifolates on enzymatic and cellular processes.

The synthesis of DL-3,3-difluoroglutamic acid[16] and its incorporation into methotrexate and folic acid analogues results in DL-β,β-difluoromethotrexate, **2**,[6] and DL-β,β-difluorofolic acid, **4**. In agreement with our previous observation that ligation of 3,3-difluoroglutamic acid to either methotrexate or folic acid leads to products with enhanced FPGS substrate properties,[4] both **2** and **4** are better substrates than MTX[6] and folic acid (Figure 3), respectively. Surprisingly, the ligation product containing β,β-difluoroglutamyl-γ-glutamate is essentially inactive as an FPGS substrate.[6] Thus, incorporation of 3,3-difluoroglutamate into a growing poly-γ-glutamyl conjugate of folic acid or methotrexate[4] or into synthetic

analogues such as **2** and **4**[6] results in enhanced ligation of a single glutamic acid followed by chain termination; i.e., 3,3-F_2Glu mediates a position-dependent enhancement or termination of FPGS-catalyzed polyglutamylation.[6]

These results are informative and suggest approaches to address more fundamental issues related to FPGS catalysis. It is clear that the presence of CF_2 in 3,3-F_2-Glu substrates vs CH_2 in glutamate substrates leads to a dramatic change in the structure of the enzyme−substrate complex during catalysis. This change is manifest by either a stimulation of catalytic activity using substrates with 3,3-F_2Glu at the C-terminus[4,6] or termination of the ligation reaction by substrates with the C-terminal sequence -3,3-F_2Glu-γ-Glu.[6] Unfortunately, very little is known about the nature of these complexes and/or their rearrangements during chain elongation. Since FPGS catalyzes the nonribosomal synthesis of a polypeptide, it is important to ascertain if the reaction proceeds via a processive or a nonprocessive mechanism. Steady-state kinetic data indicate that FPGS catalysis proceeds via an ordered, sequential mechanism with an order of substrate binding (MgATP, folate, glutamate) and product release (ADP, folate poly-γ-glutamate, P_i) which appear to rule out the sequential addition of glutamate to enzyme-bound folate, i.e., a processive mechanism. However, the published experiments were carried out under conditions where only a single glutamate was added due to the FPGS chosen for study (*Corynebacterium* sp.)[31] or substrate (aminopterin) and assay conditions used.[32] Our results indicate that 3,3-difluoroglutamate-containing conjugates of MTX and reduced folates may be useful probes for investigating the required rearrangement of enzyme−substrate complexes during the synthesis of a growing poly-γ-glutamyl peptide.

Experimental Section

General Techniques. All reactions sensitive to moisture were conducted under an atmosphere of nitrogen with oven-dried glassware, unless specified otherwise. All commercial reagents were distilled prior to use. Chloroform was distilled from CaH_2 and stored over 4 Å molecular sieves, and dichloromethane was freshly distilled from CaH_2. Triethylamine was distilled from KOH and stored over molecular sieves. Column chromatography was performed with silica gel 60 (230−400 mesh). Thin layer chromatography (TLC) was conducted on precoated silica gel plates (Kieselgel 60 F254, 0.25-mm thickness, E. Merck & Co.) and visualized by ultraviolet light and/or ninhydrin followed by heat. Melting points were obtained on a Thomas-Hoover Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AM-300 or AM-360 and reported in the following manner: chemical shift in ppm downfield from tetramethylsilane (multiplicity, integrated intensity, coupling constants in hertz, assignment). ^{13}C NMR spectra were recorded on a Bruker AM-300 or AM-360 referenced to tetramethylsilane. ^{19}F NMR were obtained on a GE Omega-500 or Bruker AM-300 and referenced to trifluoroacetic acid as an external standard. Infrared spectra were recorded on a Nicolet 5-DX spectrometer. Mass spectra and high-resolution mass spectra were performed on a Finnigan 4500 GC/MS-EICI system or on a VG analytical system, Model 70-250S. Elemental analyses were obtained from Atlantic Microlab Inc. (Norcross, GA) or at the Elemental Analysis Laboratory, Department of Chemistry, University of Michigan. Yields of fluorinated folates and antifolates were determined on the basis of the extinction coefficients for the analogous protio compounds.[33]

MTX was a generous gift of the National Cancer Institute and Immunex (Seattle, WA). Aminopterin (AMT) was obtained from Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile (J. T. Baker) was from Fisher Scientific Co. 2-Mercaptoethanol and Norit were obtained from Kodak. $RuO_2 \cdot xH_2O$ was obtained from AESAR (Ward Hill, MA). DAST was from Carbolabs (Britany, CT) and was used without further purification. The 7,8-dihydro derivatives of folates were produced by microscale dithionite reduction and repurification by repeated acid precipitation essentially as described by Hayman.[34] The pH was adjusted to 1.8 and the solution placed on ice for 3 h to allow precipitation. The pH is lower than that optimal for the precipitation of dihydrofolate; this is consistent with the report that a lower pH is required to precipitate γ-fluorofolic acid.[30] Concentrations of pteridine solutions for all experiments were standardized using published extinction coefficients.

(2*RS*,4*RS*)-DL-*erythro*,*threo*-4-Fluoroglutamic acid, **5*e*,*t*** (*rac*), was synthesized from diethylfluoromalonate and ethyl acetamidomalonate.[5] Conversion to the di-*tert*-butyl ester, **7*e*,*t*** (*rac*), and separation of the *erythro* and *threo* diastereomeric esters has been reported previously without details;[5] complete experimental details and spectral data are provided herein (supporting information). (2*S*,4*S*)-L-*threo*-4-Fluoroglutamic acid (**5*t***) was synthesized as recently described by Hudlicky.[19] The synthesis of DL-3,3-difluoroglutamic acid, **6**, is described below in detail; selected spectral data for **6** and intermediates in the synthesis of **6** have been presented in a previous communication.[16] *N*-Me,*N*-Cbz-4-aminobenzoic acid,[35] 2,4-diamino-6-(bromomethyl)pteridine (**10**),[35] and N^{10}-(trifluoroacetyl)pteroic acid (**11**)[21] were synthesized as previously described. The cyclic carbamate of 3-hydroxy-DL-prolinol, **12**, was synthesized as described by Tamaru et al.[36]

DEAE-cellulose chromatography general procedure: DE 53 (Whatman, 6 g) was washed with triethylammonium bicarbonate (TEAB) buffer (1.0 M, 2 × 50 mL), and the pH was adjusted with CO_2(g) to approximately 7.7. The DE 53 was allowed to settle and the buffer was decanted. The resulting DE 53 was suspended in TEAB buffer (0.025 or 0.3 M), and the column bed (1 × 12 cm) was poured at 4 °C. The column pH and conductivity were equilibrated with TEAB buffer (0.025 or 0.3 M). The sample was dissolved in H_2O, and the pH and conductivity were adjusted to match the equilibrating buffer. The sample was applied to the column, and the product was eluted, at 4 °C, with a linear buffer gradient (500 mL total volume, 0.025 or 0.3−1.0 M). Fractions (12 mL) were collected and analyzed by UV absorption spectroscopy. Fractions containing the desired product were pooled and evaporated to dryness. The resulting solid was dissolved in H_2O and lyophilized.

HPLC chromatography general procedure: Synthetic compounds were analyzed by reversed-phase HPLC (Vydac C_{18} column; 0.46 × 25 cm) using a Beckman/Altex system equipped with a Rheodyne Model 7125 injector, two Model 110 A Altex pumps, and a Hewlett-Packard Model 1040A detector set at 280 nm. The mobile phase consisted of eluent A (0.1 M sodium acetate, pH 5.5) and eluent B (CH_3CN), and the elution proceeded as follows at 1 mL/min.[37] The initial conditions were 4% B (0−12 min) and then increased linearly to 12.4% B (12−40 min) and run at 12.4% B (40−42 min).

(2*S*,4*S*)-4-Fluoroglutamic Acid α,γ-Di-*tert*-butyl Ester (7*t*). To a suspension of **5*t*** (0.80 g, 4.84 mmol) in $CHCl_3$ (50 mL) at −20 °C was added concentrated H_2SO_4 (0.56 mL). The solution was maintained at −20 °C, and isobutylene gas (12 mL) was condensed into the flask. The flask was closed to the atmosphere and stirred at room temperature for 96 h. The mixture was cooled to −20 °C and opened to the atmosphere. The solution was washed with 20% K_2CO_3 (100 mL), and the organic layer was separated. The aqueous layer was extracted with EtOAc (2 × 50 mL), and the combined organic layers were dried over $MgSO_4$, filtered, and evaporated in vacuo. The resulting oil was purified by silica gel chromatography (hexanes/EtOAc, 2:1) to afford **7*t*** (0.91 g; 68%) as a colorless oil: $[\alpha]^{22}_D = -8.34$ (*c* 1.1, $CHCl_3$); R_f 0.55 (hexanes/EtOAc, 2:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.10 (ddd, J = 49.5, 10.4, 1.9 Hz, 1 H, CHF), 3.55 (dd, J = 10.3, 3.3 Hz, 1 H, αCH), 2.40−1.70 (m, 2 H, CH_2); ^{13}C NMR (90 MHz, $CDCl_3$) 174.4, 169.6 (d, J = 20.2 Hz), 86.6 (d, J = 183.7 Hz), 82.5, 81.4, 51.6, 37.5 (d,

J = 21.2 Hz), 28.1 (6C); ^{19}F NMR (480 MHz, $CDCl_3$) δ −123.6 (ddd, J = 49.2, 15.1, 14.3 Hz); MS (DCI-NH_3) m/z (relative intensity) 278 ($(M + H)^+$, 75), 222 (66), 166 (23); HRMS for $C_{13}H_{24}FNO_4$ $(M + H)^+$ calcd 278.1768 found 278.1776.

N*-[4-[[[(Benzyloxy)carbonyl]methyl]amino]benzoyl]-(αS,γS)-γ-fluoroglutamic Acid α,γ-Di-*tert*-butyl Ester (8*t*).** To a solution of 4-[(*N*-Cbz)methylamino]benzoic acid (0.21 g, 0.72 mmol) in DMF (15 mL) at 0 °C were added DCC (0.25 g, 1.0 mmol) and HOBt (0.2 g, 1.44 mmol) followed by **7*t (0.2 g, 0.72 mmol). After 60 h of stirring at room temperature, the solution was cooled to 5 °C and filtered. The filtrate was evaporated to dryness, and the resulting yellow semisolid was purified by silica gel column chromatography (hexanes/EtOAc, 2:1) to afford **8*t*** (0.38 g, 97%) as a colorless oil: $[\alpha]^{24}_D$ = +11.15 (*c* 1.07, $CHCl_3$); R_f 0.40 (hexanes/EtOAc, 1:1); IR ($CHCl_3$) 1730, 1706, 1667, 1608 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 (d, J = 8.8 Hz, 2 H, Ar), 7.44−7.31 (m, 7 H, C_6H_5 and Ar), 6.85 (bd, 1 H, NH), 5.18 (s, 2 H, benzyl), 5.05−4.80 (m, 2 H, CHF and αH), 3.35 (s, 3 H, CH_3), 1.78−2.39 (m, 2 H, βCH_2), 1.51 (s, 9 H, $C(CH_3)_3$), 1.45 (s, 9 H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 168.1 (d, J = 22.4 Hz), 167.8, 166.2, 154.9, 146.3, 136.3, 130.9, 128.4 (2C), 128.0 (2C), 127.7 (2C), 125.0 (2C), 86.7 (d, J = 185.1 Hz), 83.1, 82.8, 67.6, 50.5, 37.3, 35.1 (d, J = 20.5 Hz), 28.0 (6C); ^{19}F NMR (500 MHz, $CDCl_3$) δ −113 (ddd, J = 51.6, 22.5, 21.7 Hz); UV λ_{max} (0.1 N NaOH) 267 nm; (0.1 N HCl) 266, 226 nm; MS (EI) m/z (relative intensity) 554 (M^+, 8), 471 (3), 432 (5), 415 (12), 387 (27); HRMS for $C_{29}H_{37}FN_2O_7$ (M^+) calcd 544.2585, found 544.2580. Anal. ($C_{29}H_{37}FN_2O_7$) C, H, N.

N*-[4-[Methylamino]benzoyl]-(αS,γS)-γ-fluoroglutamic Acid α,γ-Di-*tert*-butyl Ester (9*t*).** To a solution of **8*t (0.37 g, 0.70 mmol) in MeOH (20 mL) was added $Pd(OH)_2$ (0.047 g), and the mixture was shaken under hydrogen (50 psi) for 48 h. The solution was filtered over silica gel followed by decolorizing charcoal (Norit). The filtrate was evaporated to dryness, and the resulting semisolid was purified by silica gel column chromatography (hexanes/EtOAc, 1:1) to afford **9*t*** (0.26 g, 99%). **9*t*** was crystallized from MeOH/H_2O: mp 156−158 °C; $[\alpha]^{22}_D$ = +19.45 (*c* 1.35, $CHCl_3$); R_f 0.34 (hexanes/EtOAc, 1:1); IR (KBr) 3393, 1744, 1627, 1611 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (d, J = 8.7 Hz, 2 H, Ar), 6.87 (d, J = 8.9 Hz, 1 H, NH), 6.59 (d, J = 8.7 Hz, 2 H, Ar), 4.95 (m, 2 H, CHF and αH), 2.87 (s, 3 H, CH_3), 2.25−2.52 (m, 2 H, CH_2), 1.49 (s, 9 H, $C(CH_3)_3$), 1.45 (s, 9 H, $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7, 167.6 (d, J = 20.1 Hz), 166.8, 152.0, 128.8 (2C), 121.8, 111.3 (2C), 86.6 (d, J = 185.0 Hz), 83.0, 82.6, 50.1, 35.4 (d, J = 20.7 Hz), 30.3, 28.0 (6C); ^{19}F NMR (480 MHz, $CDCl_3$) δ −112.63 (ddd, J = 48.9, 20.7, 19.3 Hz); UV λ_{max} (0.1 N NaOH) 291 nm; (0.1 N HCl) 227 nm; MS (EI) m/z (relative intensity) 410 (12, M^+), 337 (3), 298 (5), 281 (9), 253 (15), 134 (100); HRMS for $C_{21}H_{31}FN_2O_5$ (M^+) calcd 410.2217, found 410.2209. Anal. ($C_{21}H_{31}FN_2O_5$) C, H, N.

N*-(4-Amino-4-deoxy-10-methylpteroyl)-(αS,γS)-γ-fluoroglutamic Acid (1*t*).** To a solution of **10** (0.12 g, 0.38 mmol) in DMAC (2 mL) was added a solution of **9*t (0.060 g, 0.16 mmol) in DMAC (2 mL) at room temperature. The mixture was heated at 55 °C for 4 h, cooled to room temperature, and stirred for 24 h. TLC (R_f 0.80; butanol/H_2O/pyridine, B/W/P, 30:24:26) indicated the reaction was complete and the solution was evaporated to dryness. The resulting solid was dissolved in TFA (3 mL) and stirred at room temperature for 3 h. The mixture was evaporated to dryness, and the resulting solid was triturated with Et_2O and filtered. The crude product was purified by DEAE-cellulose column chromatography. The desired product, **1*t***, eluted during a 0.025 M−1.0 M TEAB, pH 7.7, linear gradient. Fractions containing pure **1*t***, identified by UV spectra, were pooled and evaporated to dryness. The resulting solid was dissolved in H_2O and lyophilized to afford **1*t*** (0.11 mmol) as the triethylamine salt in 69% yield: mp >300 °C; R_f 0.57 (B/W/P, 30:24:26); ^{1}H NMR (300 MHz, D_2O) δ 8.43 (s, 1 H, C-7H), 7.60 (d, J = 8.7 Hz, 2 H, Ar), 7.08 (d, J = 8.8 Hz, 2 H, Ar), 4.70 (s, 2 H, benzyl), 3.20 (s, 3 H, CH_3), 3.03 (q, 12 H, $N(CH_2CH_3)_3$ × 2), 2.60−2.40 (m, 2 H, βCH_2), 1.1 (t, 18 H, $N(CH_2CH_3)_3$ × 2), CHF and α-CH resonances were obscured by HOD peak but were observed at 5.01 and 4.51 ppm in DMSO-d_6; ^{13}C NMR (90 MHz, D_2O) δ 178.2, 177.0 (d, J = 20.5 Hz), 168.9, 162.6, 161.8, 153.2, 151.3, 149.0, 147.9, 128.7 (2C), 121.8, 120.1, 111.4 (2C), 88.2 (d, J = 182.3 Hz), 54.5, 52.0, 45.9, 42.4, 38.8, 35.7 (d, J = 21.6 Hz), 8.7; ^{19}F NMR (480 MHz, D_2O) δ −115.30 (ddd, J = 50.0, 18.0, 16.9 Hz); UV λ_{max} (0.1 N NaOH) 258, 302, 371 nm; (0.1 N HCl) 307, 242, 346 (sh) nm; MS (FAB^-, triethanolamine) for $C_{20}H_{21}FN_8O_5$ m/z (relative intensity) 471 (M − 1, 43.1); reversed phase HPLC t_R = 37.3 min.

N*-Pteroyl-(αS,γS)-γ-fluoroglutamic Acid (3*t*).** To a solution of **11** (0.13 g, 0.32 mmol) in DMF (7 mL) cooled to 0 °C were added DCC (0.098 g, 0.48 mmol) and HOBt (0.086 g, 0.64 mmol). The mixture became homogeneous as it warmed to room temperature. The resulting solution was cooled to 0 °C and a solution of **7*t (0.089 g, 0.32 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at room temperature for 68 h and filtered. The filtrate was evaporated to dryness, and the resulting solid was dissolved in EtOAc/DMF (90/10, 100 mL), washed with 0.5 N $NaHCO_3$ (1 × 50 mL), H_2O (1 × 50 mL), 0.5 N H_2SO_4 (1 × 50 mL), H_2O (1 × 50 mL), 0.5 N $NaHCO_3$ (1 × 50 mL), and saturated NaCl (1 × 50 mL), and dried over Na_2SO_4. The solution was filtered and evaporated to dryness, and the resulting solid was dissolved in TFA (10 mL) at 0 °C. After stirring for 30 min at 0 °C, the mixture was diluted with $CHCl_3$ (50 mL) and evaporated to dryness. The resulting yellow solid was dissolved in 0.1 M piperidine (25 mL) and stirred for 3 h at 15 °C. The turbid solution was centrifuged (20 min, 1500*g*), the supernatant was decanted, and the pH was adjusted to 2 with 2 N HCl. The solution was stored at 4 °C for 12 h to provide an opaque suspension. Centrifugation provided an orange pellet that was washed with H_2O (2 × 25 mL) and recentrifuged. The resulting yellow pellet was dried in vacuo to afford **3*t*** (0.083 g, 56%): ^{1}H NMR (360 MHz, DMSO-d_6/D_2O exchange) δ 8.60 (s, 1 H, C7-H), 7.65 (d, J = 8.7, 2 H, Ar), 6.62 (d, J = 8.7, 2 H, Ar), 4.89 (m, 1 H, CHF), 4.50−4.40 (m, 3 H, αCH and benzylic CH_2), 2.45−2.15 (m, 2 H, βCH_2); ^{13}C NMR (90 MHz, DMSO-d_6) δ 173.4, 171.0 (d, J = 22.6 Hz), 167.1, 161.6, 156.3, 153.9, 151.2, 149.0 (2C), 131.5, 129.4 (2C), 128.1, 121.3, 111.6 (2C), 86.5 (d, J = 182.0 Hz), 48.7, 46.0, 33.6 (d, J = 20.01 Hz); ^{19}F NMR (500 MHz, DMSO-d_6) δ −114.8 (bm); UV λ_{max} (0.1 N NaOH) 255, 281, 362 nm; MS (FAB^-, DTT/DTE) for $C_{19}H_{18}FN_7O_6$ m/z (relative intensity) 458 (M − 1, 100); reversed phase HPLC, t_R = 24.3 min.

6-Oxo-1-aza-3-oxabicyclo[3.3.0]octan-2-one (13). To a solution of DMSO (0.36 mL, 5.1 mmol) in CH_2Cl_2 (25 mL) cooled to −55 °C was added trifluoroacetic anhydride (0.54 mL, 3.83 mmol) in CH_2Cl_2 (2.1 mL), maintaining the temperature below −50 °C. A white precipitate formed, and the mixture was stirred for 10 min. Alcohol **12** (0.36 g, 2.55 mmol), dissolved in $CHCl_3$ (15 mL), was added slowly to the reaction mixture, maintaining the temperature between −50 °C and −55 °C. After the addition of **12** the reaction mixture was stirred at −55 °C for 30 min. Triethylamine (1.02 mL, 7.32 mmol) was added dropwise to the mixture, maintaining the temperature between −50 °C and −55 °C. After stirring at room temperature for 16 h, the mixture was concentrated in vacuo, and the resulting yellow oil was dissolved in CH_2Cl_2 (100 mL) and washed with H_2O (50 mL). The aqueous phase was extracted with CH_2Cl_2 (4 × 50 mL), and the combined organic phases were washed with saturated NaCl (2 × 50 mL). Additional product was obtained from the aqueous phase by saturation with NaCl followed by extraction with CH_2Cl_2 (4 × 100 mL). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated in vacuo. The crude oil was purified by silica gel chromatography (EtOAc/hexanes/CH_3CN, 3:1:1) to afford **13** (0.26 g, 73%) as a white crystalline solid: mp 98−99 °C; R_f 0.74 (EtOAc/hexanes/CH_3CN, 3:1:1); IR ($CHCl_3$) 1763, 1706 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.55 (t, 1 H, J = 9.4 Hz, C4-H), 4.40 (dd, 1 H, J = 3.4, 3.4 Hz, C4-H), 4.31 (m, 1 H, C8-H), 3.93 (dd, 1 H, J = 3.3, 3.5 Hz, C5-H), 3.60 (m, 1 H, C8-H), 2.56 (m, 2 H, C7-H_2); ^{13}C NMR (90 MHz, $CDCl_3$) δ 212.6, 161.4, 64.5, 61.3, 43.8, 36.4; MS (EI) m/z (relative intensity) 142 ($(M + H)^+$, 10), 141 (M^+, 17), 113 (100), 97 (59), 86 (96); HRMS for $C_6H_7NO_3$ (M^+) calcd 141.0426, found 141.0434. Anal. ($C_6H_7NO_3$) C, H, N.

6,6-Difluoro-1-aza-3-oxabicyclo[3.3.0]octan-2-one (14). To a solution of **13** (0.10 g, 0.71 mmol) in CH_2Cl_2 (15 mL) was added DAST (0.30 mL, 2.10 mmol) at −78 °C and the solution was stirred at room temperature for 36 h. Ice and CH_2Cl_2 (30 mL) were added to the solution, and the organic and aqueous phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL), and the combined organic phases were dried over $MgSO_4$, filtered, and concentrated in vacuo, providing **14** (0.073 g, 64%) as a yellow oil: R_f 0.90 (EtOAc/hexanes, 4:1, developed by I_2); IR ($CHCl_3$) 1760 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.52 (m, 2 H, C4-H_2), 4.08 (m, 1 H, C5-H_2), 3.89−3.47 (m, 2 H, C8-H_2), 2.46 (m, 2 H, C8-H_2); ^{13}C NMR (90 MHz, $CDCl_3$) δ 160.6, 126.7 (t, J = 256.6 Hz), 62.2 (t, J = 26.1 Hz), 61.6, 43.2, 34.5 (t, J = 24.9 Hz); MS (EI) m/z (relative intensity) 163 (M^+, 25), 119 (12), 99 (19); HRMS for $C_6H_7F_2NO_2$ (M^+) calcd 163.0445, found 163.0452. Compound **14** was converted to **15** without further purification.

DL-*N*-(*tert*-Butoxycarbonyl)-3,3-difluoro-2-(hydroxymethyl)pyrrolidine (DL-(*N*-Boc)-3,3-difluoroprolinol) (15). A solution of difluorooxazolidinone **14** (0.10 g, 0.61 mmol) in 6 N HCl (3 mL) was heated at reflux temperature for 18 h. The solution was evaporated in vacuo, providing crude 3,3-difluoroprolinol as a light brown oil (0.10 g). To a suspension of the crude oil in $CHCl_3$ (1.5 mL) was added 0.36 M $NaHCO_3$ (1.5 mL), NaCl (0.06 g), and $(Boc)_2O$ (0.16 g, 0.70 mmol). The solution was heated at reflux temperature for 3 h and cooled to room temperature. The aqueous and organic phases were separated, and the aqueous phase was extracted with $CHCl_3$ (4 × 30 mL). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated in vacuo to give **15** as a yellow oil (0.13 g, 83% from **14**): R_f 0.90 ($CHCl_3$/EtOAc, 9:1); IR ($CHCl_3$) 3473, 1686 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.00−3.65 (m, 3 H, C2-H and C5-H_2), 3.5 (m, 2 H, CH_2O), 2.44−2.21 (m, 2 H, C4-H_2), 1.45 and 1.60 (two singlets, 9 H, $C(CH_3)_3$); ^{13}C NMR (90 MHz, $CDCl_3$) δ 155.3, 127.3 (t, J = 269.9 Hz), 81.2, 64.2 (t, J = 28.1 Hz), 61.3, 43.4, 33.2 (t, J = 23.7 Hz), 28.5 (3C); ^{19}F NMR (282 MHz, $CDCl_3$) δ (two rotamers) −17.5 (dm, J = 231.5 Hz) and −22.4 (d, J = 235.9 Hz), −35.9 (d, J = 234.6 Hz) and −36.4 (d, J = 236.7 Hz); MS (CI-NH_3) m/z (relative intensity) 238 ($(M + H)^+$, 9), 199 (37), 182 (100), 138 (26); HRMS for $C_{10}H_{17}F_2NO_3$ $(M + H)^+$ calcd 238.1247, found 238.1249. Compound **15** was converted to **16** without further purification. An analytical sample was obtained by silica gel column chromatography (hexanes/EtOAc, 3:1). Anal. ($C_{10}H_{17}F_2NO_3 \cdot 0.1H_2O$) C, H, N.

DL-*N*-(*tert*-Butoxycarbonyl)-3,3-difluoroproline Methyl Ester (16). A solution of **15** (0.13 g, 0.41 mmol) dissolved in EtOAc (3 mL) was added to a solution of $RuO_2 \cdot xH_2O$ (0.020 g, catalytic) in aqueous 10% $NaIO_4$ (3.5 mL, 1.6 mmol) at room temperature. The biphasic solution was stirred vigorously at room temperature for 3 h, allowing thorough mixing between phases. The organic and aqueous phases were separated, and the aqueous phase was extracted with EtOAc (4 × 30 mL). *i*-PrOH (5 mL) was added to the combined organic phases, and the mixture was stirred at room temperature for 3 h and was dried over $MgSO_4$, filtered through a plug of Celite, and evaporated in vacuo. The resulting crude acid (0.13 g) was not purified. ^1H NMR indicated no starting material remained and was consistent with the formation of the corresponding acid, R_f 0.10 ($CHCl_3$/EtOAc, 9:1, bromocresol green-positive). To a solution of the crude acid in Et_2O (15 mL) was added CH_2N_2 (3.0 mL, 1.53 mmol), and the mixture was stirred at 0 °C for 1 h. Glacial acetic acid (0.05 mL) was added to the reaction mixture and the solution stirred at room temperature for 25 min. The solvent was removed under a weak vacuum (20 °C, 340 mbar) (**CAUTION**! **16** is volatile under high vacuum) and the residue was purified by silica gel column chromatography (hexanes/EtOAc, 2:1) to afford **16** (0.10 g, 93% from **15**) as a colorless oil: R_f 0.70 (hexanes/EtOAc, 2:1); IR ($CHCl_3$) 1754, 1706, 1704 (sh) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.53 (m, 2 H, αCH), 3.70−3.50 (m, 5 H, δCH_2 and CH_3), 2.50−2.35 (m, 2 H, γCH_2), 1.50 and 1.45 (two singlets, 9 H, $C(CH_3)_3$); ^{13}C NMR (90 MHz, $CDCl_3$) (two rotamers) δ 167.8, 153.6 and 153.0, 126.5 (t, J = 254.2 Hz) and 125.9 (t, J = 260.0 Hz), 81.0, 65.0 (t, J = 30.3 Hz) and 64.4 (t, J = 30.3 Hz), 52.8 and 52.6, 43.3 and 42.8, 33.3 (t, J = 29.9 Hz) and 32.7 (t, J = 23.1 Hz), 28.2 (3C) and 28.1 (3C); ^{19}F NMR (282 MHz, $CDCl_3$) (two rotamers) δ −18.6 (d, J = 235.8 Hz) and −18.8 (d, J = 236.1 Hz), −30.2 (d, J = 236.9 Hz) and −32.6 (d, J = 236.9 Hz); MS (CI-NH_3) m/z (relative intensity) 283 ($(M + NH_4)^+$, 7), 266 ($(M + H)^+$, 4), 227 (100), 166 (28), 136 (79); HRMS for $C_{11}H_{17}F_2NO_4$ $(M + H)^+$ calcd 266.1204, found 266.1198. Anal. ($C_{11}H_{17}F_2NO_4$) H, N; C: calcd, 49.81; found, 49.05.

DL-3,3-Difluoroglutamic Acid (6). A solution of **16** (0.25 g, 0.94 mmol) in EtOAc (10 mL) was added to a solution of $RuO_2 \cdot xH_2O$ (0.050 g, catalytic) in aqueous 10% $NaIO_4$ (8.6 mL) at room temperature. The biphasic solution was stirred vigorously at room temperature to allow for thorough mixing between phases. Additional aliquots of 10% $NaIO_4$ (5 × 2 mL) were added to maintain a yellow-colored solution, and the reaction mixture was stirred at room temperature for 288 h. The organic and aqueous phases were separated, and the aqueous phase was extracted with EtOAc (3 × 50 mL). *i*-PrOH (5 mL) was added to the combined organic phases, and the mixture was stirred at room temperature for 3 h, dried over $MgSO_4$, filtered through a plug of Celite, and evaporated in vacuo, providing a dark oil **17** (0.17 g). A portion of the crude pyroglutamate **17** was crystallized for analysis: mp 62−63 °C; R_f 0.40 (hexanes/EtOAc, 2:1); IR (KBr) 2950, 1721, 1658 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.90−4.80 (d, J = 17.4 Hz, 1 H, αCH), 3.88 (s, 3 H, CH_3), 3.29−2.98 (m, 2 H, γCH_2), 1.45 (s, 9 H, $C(CH_3)_3$); ^{13}C NMR (90 MHz, $CDCl_3$) δ 166.1, 165.6, 148.0, 118.8 (t, J = 252.5 Hz), 85.2, 66.4 (t, J = 31.0 Hz), 53.4, 41.3 (t, J = 24.5 Hz), 27.8 (3C); ^{19}F NMR (282 MHz, $CDCl_3$) δ −13.6 (dm, J = 240.8 Hz), −31.1 (dd, J = 240.4 Hz, J = 12.0 Hz); MS (CI-NH_3) m/z (relative intensity) 297 ($(M + NH_4)^+$, 36), 280 ($(M + H)^+$, 2), 197 (49), 177 (42), 136 (100); HRMS for $C_{11}H_{15}F_2NO_5$ $(M + NH_4)^+$ calcd 297.1262, found 297.1259. Anal. ($C_{11}H_{15}F_2NO_5$) C, H, N.

A solution of crude **17** (0.032 g) in 12 N HCl (3 mL) was heated at reflux temperature for 2 h. The solution was evaporated to dryness, providing 0.030 g of an off-white solid. The crude solid **6** was dissolved in ddH_2O and purified by anion-exchange column chromatography (BioRad AG 1-X8 200−400 mesh, acetate form, 11 mL of wet resin = 18.7 mequiv). The column was eluted with ddH_2O (110 mL), 1 N HOAc (110 mL), and 2 N HOAc (110 mL), and fractions (10 mL) were collected. Fractions 16−18 (1N HOAc) were pooled, concentrated in vacuo, and crystallized ($EtOH/H_2O$) to afford **6** (0.013 g, 40% from **16**): mp 171−174 °C, R_f 0.35 (B/W/P, 30:24:26); ^1H NMR (300 MHz, D_2O) δ 4.65−4.51 (dd, J = 3.8, 3.7 Hz, 1 H, αCH), 3.60−3.36 (m, 2 H, γCH_2); ^{13}C NMR (90 MHz, D_2O) δ 170.7 (d, J = 11.8 Hz), 167.2 (d, J = 7.1 Hz), 119.2 (t, J = 248.3 Hz), 57.5 (dd, J = 19.9, 19.7 Hz), 39.9 (t, J = 24.9 Hz); ^{19}F NMR (282 MHz, D_2O) δ −22.1 (dm, J = 253.3 Hz), −27.1 (d, J = 256.4 Hz). Anal. ($C_5H_7F_2O_4$) C, H, N.

DL-3,3-Difluoroglutamic Acid α,γ-Di-*tert*-butyl Ester (18). To a suspension of **6** (0.047 g, 0.26 mmol) in freshly distilled $CHCl_3$ (3 mL) at −20 °C was added concentrated H_2SO_4 (0.028 mL) slowly by pipette. The solution was maintained at −20 °C, and isobutylene gas (3 mL) was condensed into the flask. The vessel was sealed, and after stirring at room temperature for 72 h the solution became homogeneous. After 120 h the mixture was cooled to −20 °C, opened, and washed with 20% K_2CO_3 (50 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL), and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated in vacuo. The resulting oil was purified by silica gel column chromatography (hexanes/EtOAc, 10:3) to afford **18** (0.068 g, 89% yield) as a colorless oil: R_f 0.33 (hexanes/EtOAc, 10:3); ^1H NMR (300 MHz, $CDCl_3$) δ 4.06 (t, J = 13.6 Hz, 1 H, αCH), 3.02 (m, 2 H, γCH_2), 1.48 (s, 9 H, $C(CH_3)_3$), 1.45 (s, 9 H, $C(CH_3)_3$); ^{13}C NMR (90.0 MHz, $CDCl_3$) δ 168.7, 166.1, 120.6 (t, J = 248.3 Hz), 82.6, 81.9, 58.4 (t, J = 26.1 Hz), 40.6 (t, J = 26.7 Hz), 27.9 (6C); ^{19}F NMR (470 MHz, $CDCl_3$) δ −27.2 (ddd, J = 253.2, 15.1, 11.8 Hz), −27.8 (ddd, J = 253.2, 15.1, 12.2 Hz); MS (DCI-NH_3) m/z (relative intensity) 296 ($(M + H)^+$, 25), 240 (32), 184 (14); HRMS for $C_{13}H_{23}F_2NO_4$ $(M + H)^+$ calcd 296.1673 found 296.1674.

***N*-Pteroyl-DL-β,β-difluoroglutamic Acid (4).** To a solution of **11** (0.080 g, 0.20 mmol) in DMF (2 mL) was added HOBt (0.053 g, 0.39 mmol), EDC (0.075 g, 0.39 mmol), and **18** (0.058

g, 0.20 mmol) in DMF (2 mL) at room temperature. After 72 h the solution was evaporated to dryness, and the resulting solid was dissolved in EtOAc/DMF (90/10, 100 mL), washed with 0.5 N $NaHCO_3$ (1 × 50 mL), H_2O (1 × 50 mL), 0.5 N H_2SO_4 (1 × 50 mL), H_2O (1 × 50 mL), 0.5 N $NaHCO_3$ (1 × 50 mL), and saturated NaCl (1 × 50 mL), and dried over Na_2SO_4. The solution was filtered and evaporated to dryness, and the resulting solid was dissolved in TFA (10 mL) at 0 °C. After stirring for 30 min at 0 °C the mixture was diluted with $CHCl_3$ (50 mL) and evaporated to dryness. The resulting yellow solid was dissolved in 0.1 M piperidine (25 mL) and stirred for 3 h at 15 °C. The pH of the solution was adjusted to 2 with 2 N HCl and was stored at 4 °C for 12 h to provide an opaque suspension. Centrifugation provided an orange pellet that was washed with H_2O (2 × 25 mL) and the suspension recentrifuged. The resulting pellet was dried in vacuo to afford crude **4**. The solid was purified by DEAE-cellulose column chromatography. The product eluted during a 0.3−1.0 M TEAB, pH 7.7, linear gradient. Fractions containing pure **4** (0.5−0.8M TEAB), identified by UV spectra, were pooled and evaporated to dryness. The resulting solid was dissolved in H_2O and lyophilized to afford **4** (0.069 mmol) as the triethylamine salt in 35% yield: mp >280 °C dec; ^{1}H NMR (360 MHz, D_2O) δ 8.46 (s, 1 H, C7-H), 7.42 (d, J = 8.7 Hz, 2 H, Ar), 6.36 (d, J = 8.7 Hz, 2 H, Ar), 4.75 (t, 1 H, αCH), 4.29 (s, 2 H, C9-H_2), 3.11 (q, H, N(CH_2CH_3)), 2.90 (t, 2 H, γCH_2), 1.1 (t, N(CH_2CH_3)); ^{13}C NMR (90 MHz, D_2O) δ 174.3, 172.4, 169.1, 164.6, 154.0, 152.7, 150.5, 148.7, 148.4, 129.1 (2C), 126.6, 121.1 (t, J = 248.1 Hz), 120.2, 115.1, 111.6 (2C), 59.2 (t, J = 26.3 Hz), 58.6, 47.2, 46.6, 45.0, 42.6 (t, J = 24.4 Hz), 42.3, 18.3, 10.6, 8.2; ^{19}F NMR (470 MHz, D_2O) −24.67 (m); UV λ_{max} (0.1 M NaOH) 364, 286, 255 nm; MS (FAB^-, DTT/DTE) for $C_{19}H_{17}F_2N_7O_6$ m/z (relative intensity) 476 ($(M - H)^-$, 7); MS (FAB^+, DTT/DTE) (relative intensity) 588 ($(M + Et_3N)^+$, 8); reversed phase HPLC t_R = 29.3 min.

Biochemical Techniques. Analytical HPLC of Biosynthetic Products. Performed on a Rainin Instruments HPLC system using the Dynamax controller and data capture module run on a Macintosh computer.[4] Eluant was monitored at 280 and 254 nm. RP-HPLC was performed on a C_{18} column (0.4 × 25 cm; Rainin Microsorb, 5 μm) at ambient temperature; the column was eluted isocratically at 1 mL/min using 0.1 M sodium acetate, pH 5.5, containing 7.5% acetonitrile according to Nimec and Galivan[37] unless otherwise noted. Anion-exchange HPLC was performed on an Ultrasil AX column (0.4 × 25 cm; Beckman) at 45 °C eluted isocratically at 0.8 mL/min with 35 or 85 mM sodium phosphate, pH 3.3; the concentration required to obtain a constant elution time (7.5 min) for MTX depended on column age. Separation of polyglutamate products was performed on the anion-exchange column using a linear gradient.[4]

Cell Lines. The human T-lymphoblastic leukemia cell line CCRF-CEM[38] was the primary screen for drug effects and was the source of tumor enzymes. Routine culture of these lines was as described.[39] CCRF-CEM was verified to be negative for Mycoplasma contamination during the course of these studies using the GenProbe test kit.

Enzymes and Assays. Rat liver FPGS was purified as described.[40] FPGS and DHFR from the human T-lymphoblastic leukemia cell line CCRF-CEM[38] were partially purified as described.[41,42] CPG2 was obtained from Dr. Roger Sherwood (PHLS Centre for Applied Microbiology and Research, Porton Down, England). The FPGS assay method[40,43] 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. Rat liver FPGS assay conditions were exactly as described.[40] CCRF-CEM FPGS assays conditions[44] 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 one or two experiments to avoid absorption of atmospheric CO_2 which can lead to FPGS kinetic constants that can be in error by as much as a factor of 2.[44] DHFR activity was assayed spectrophotometrically as described.[41] Standard assays contained 100 mM Tris-HCl, pH 7.0, 150 mM KCl, 20 μM dihydrofolate, 20 mM 2-mercaptoethanol, and 50 μM NADPH. CPG2 was assayed spectrophotometrically using MTX as the substrate.[22]

Kinetic Data Analysis. FPGS kinetic data were quantitated using the nonlinear curve-fitting program of SigmaPlot (Jandel Scientific, Corte Madera, CA). Kinetic constants for FPGS substrates were determined by fitting to the rectangular hyperbola function; initial estimates of parameters were based on visual inspection of kinetic data. Inhibitory potency was measured by adding increasing concentrations of an antifolate to standard DHFR assays and measuring the remaining activity.

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Supporting Information Available: Synthesis of **3*e,t*** (*rac*), **3*e*** (*rac*), and **3*t*** (*rac*) with full experimental details (2 pages). Ordering information is given on any current masthead page.

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