5-Deazafolate Analogues with a Rotationally Restricted Glutamate or Ornithine Side Chain: Synthesis and Binding Interaction with Folylpolyglutamate Synthetase

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Rotationally restricted analogues of 5-deazapteroyl-L-glutamate and (6*R*,6*S*)-5-deaza-5,6,7,8-tetrahydropteroyl-L-glutamate with a one-carbon bridge between the amide nitrogen and the 6′-position of the *p*-aminobenzoyl moiety were synthesized and tested as substrates for folylpolyglutamate synthetase (FPGS), a key enzyme in folate metabolism and an important determinant of the therapeutic potency and selectivity of classical antifolates. The corresponding bridged analogues of 5-deazapteroyl-L-ornithine and (6*R*,6*S*)-5-deaza-5,6,7,8-tetrahydropteroyl-L-ornithine were also synthesized as potential inhibitors. Condensation of diethyl L-glutamate with methyl 2-bromomethyl-4-nitrobenzoate followed by catalytic reduction of the nitro group, reductive coupling with 2-acetamido-6-formylpyrido[2,3-*d*]pyrimidin-4(3*H*)-one in the presence of dimethylaminoborane, and acidolysis with HBr/AcOH yielded 2-L-[5-[*N*-(2-acetamido-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methylamino]-2,3-dihydro-1-oxo-2(1*H*)-isoindolyl]glutaric acid (**1**). When acidolysis was preceded by catalytic hydrogenation, the final product was the corresponding (6*R*,6*S*)-tetrahydro derivative **2**. A similar sequence starting from methyl N^{δ}-benzyloxycarbonyl-L-ornithine led to 2-L-[5-[*N*-(2-amino-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)-methylamino]-2,3-dihydro-1-oxo-2(1*H*)-isoindolyl]-5-aminopentanoic acid (**3**) and the (6*R*,6*S*)-tetrahydro derivative **4**. Compounds **3** and **4** were powerful inhibitors of recombinant human FPGS, whereas **1** and **2** were exceptionally efficient FPGS substrates, with the reduced compound **2** giving a K_m (0.018 μM) lower than that of any other substrate identified to date. (6*R*,6*S*)-5-Deazatetrahydrofolate, in which the side chain is free to rotate, was rapidly converted to long-chain polyglutamates. In contrast, the reaction of **1** and **2** was limited to the addition of a single molecule of glutamic acid. Hence rotational restriction of the side chain did not interfere with the initial FPGS-catalyzed reaction and indeed seemed to facilitate it, but the ensuing γ-glutamyl adduct was no longer an efficient substrate for the enzyme.

Introduction

Folylpolyglutamate synthetase (FPGS) plays a critical role in endogenous folate metabolism as well as in the cellular pharmacology of classical antifolates.[1–6] Intracellularly formed γ-oligoglutamyl conjugates of natural reduced folates such as 5,10-methylenetetrahydrofolate or 10-formyltetrahydrofolate generally bind better than the parent monoglutamates to their cognate enzymes and also are better retained in the cell. The chain length of each of the various reduced folate species in a cell is regulated by the concerted action of FPGS and a second enzyme, folylpolyglutamate hydrolase (FPGH), which resides in lysosomes.[7–9] Thus folate homeostasis is maintained via a combination of FPGS activity in the cytoplasmic compartment and FPGH activity in the lysosomal compartment. A similar critical role is played by FPGS and FPGH in the pharmacology of "classical" antifolates whose biochemical action and therapeutic selectivity both depend on preferential metabolism to long-chain oligoglutamates in tumor versus host tissues. FPGS was suggested to be a potential therapeutic target a number of years ago[10] on the basis of a seminal paper on purine and thymidine auxotrophy in FPGS-deficient Chinese hamster ovary cells,[11] and this concept was recently reinforced by the finding that antisense down-regulation of cellular FPGS activity leads to an overall loss of cellular reduced folates, resulting in decreased thymidylate synthesis.[12]

Because of the important role of FPGS in the pharmacologic action of classical antifolates, and more specifically in the therapeutic selectivity of these drugs and the development of resistance,[4–6] several early studies sought to broadly identify the structural requirements for binding of folates and folate antagonists to the active site of the enzyme.[13] More focused studies by several groups showed that isosteric modification of the basic folyl/antifolyl structure depicted in Figure 1 is well-tolerated at the 5- and 8-positions of the B-ring,[14–17] in the 9,10-bridge,[14] and in the phenyl ring,[17e] as are a five-membered B-ring[18] and even elimination of the B-ring by removal of the C7 and N8 ring atoms.[19] In contrast, bulky groups in the so-called "bay region" formed by C7, C9, N10, and the *ortho* position of the phenyl ring are detrimental for binding.[20–22] With at least some of these "bay region" analogues,[20] inefficient polyglutamation has been found to be due to an unusual effect involving changes in V_{max} rather than K_m.

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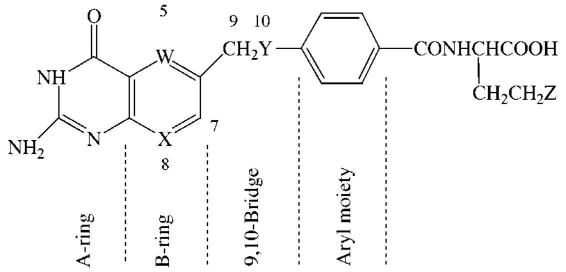
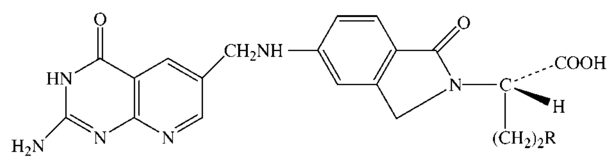


Figure 1. General structure of folyl and antifolyl substrates (Z = COOH) and inhibitors (Z = non-COOH) of FPGS. The B-ring can be five-membered six-membered and can be aromatic, reduced, or cleaved. The aryl moiety can be carbocyclic or heterocyclic and can be substituted or unsubstituted. The 4-oxo group can be replaced by 4-NH_2 and the 2-NH_2 group by 2-H or 2-Me.

Of all the molecular specificity requirements for FPGS binding by folyl substrates, the most critical for substrate activity appears to be the distance between the amide nitrogen and the γ-carboxyl group in the side chain. With rare but important exceptions,[13a] catalysis of the addition of L-glutamic acid is not observed unless the terminal carboxyl in the folyl substrate is separated from the α-carbon by precisely two CH_2 groups, and indeed, when the distance between the α-carbon and the γ-carboxyl is sufficiently long, inhibition may be observed.[23] Not surprisingly, a number of folyl analogues in which the γ-carboxyl group itself is modified are potent inhibitors, in some cases with a K_i in the nanomolar range. To date, the best inhibitors all contain an ornithine side chain,[13b,d,e,14d,24−26] though analogues containing homocysteic acid[13b,e,24b,d,27] or 2-amino-4-phosphonobutanoic acid[13e,24b,d,28] are also fairly active. It has been speculated that compounds with a sulfonate or phosphonate group in the side chain bind to the folyl binding site, whereas those containing ornithine may act as bisubstrate analogues, in that the terminal amino group occupies the binding site for the incoming glutamic acid cosubstrate.[13c] Interestingly, recent evidence indicates that the terminal amino group in ornithine analogues needs to be protonated in order for strong inhibition to occur.[29] This would be consistent with a model in which the active site contains a positive center that can interact with the negatively charged γ-carboxyl group of the folyl substrate and a negative center that can interact with the positively charged α-amino group of the incoming L-glutamate cosubstrate.

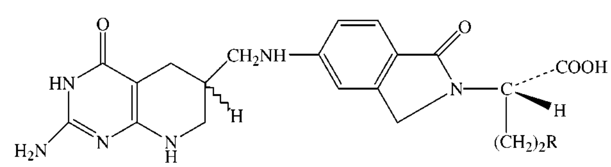
Analogues with other types of terminal modification, such as a very weakly basic alkylsulfoximine[30a] or histidine moiety[30b] or chain branching at the γ-position,[31] are very weak inhibitors. On the other hand, binding is moderately retained upon replacement of the γ-carboxyl group by an acidic γ-tetrazole ring.[32] Replacement of glutamate by β,β-difluoroglutamate,[33a,b] but not other fluorinated glutamic acids,[33b,c] actually enhances substrate activity. Of note in the context of the present work, elongation of β,β-difluoromethotrexate ceases abruptly at the diglutamate stage.[33a] In a different approach to the design of FPGS inhibitors, an interesting phosphonate dipeptide mimic of the transition state for the addition of glutamic acid to methotrexate was found to be 26-fold more inhibitory than the corresponding ornithine derivative, though unfortunately the high negative charge on the phosphapeptide side chain impeded transport across the cell membrane.[35]

As part of a broader program of structure−activity correlation involving substrates and inhibitors of FPGS, we have previously synthesized analogues modified in the region comprising the α-carbon and its flanking CONH and α-carboxyl groups.[34] In one of these analogues for example, the amide carbonyl group was replaced by CH_2.[34a] In another, the $(CH_2)_2COOH$ side chain was moved from its normal position on the α-carbon so that it resided on the amide nitrogen;[34d] in yet another, the amide nitrogen was linked to the β-carbon of glutaric acid.[13a] Binding to FPGS was drastically curtailed in all three instances, and we proposed that this could be due to a disruption of the ability of the CONH group to participate in hydrogen bonding. In the present study we were interested in addressing the question of how the binding of compounds with either a glutamate or ornithine side chain would be influenced by preventing free rotation of the bond between the phenyl ring and the CONH group. To this end, we synthesized the rotationally restricted glutamate-type analogues **1** and **2** as putative FPGS substrates and the ornithine-type analogues **3** and **4** as putative inhibitors. The 5-deazapteroyl derivatives were chosen because previous experience with FPGS inhibitors containing this ring system indicated excellent binding to the enzyme.[24d] Compounds **3** and **4** are the first reported examples of such rotationally restricted compounds in which the side chain is ornithine. While a handful of glutamate analogues with restricted rotation about the amide bond are known,[36−38] the interaction of these compounds with FPGS has been characterized in only one instance.[37]



1: R = COOH

3: R = CH_2NH_2



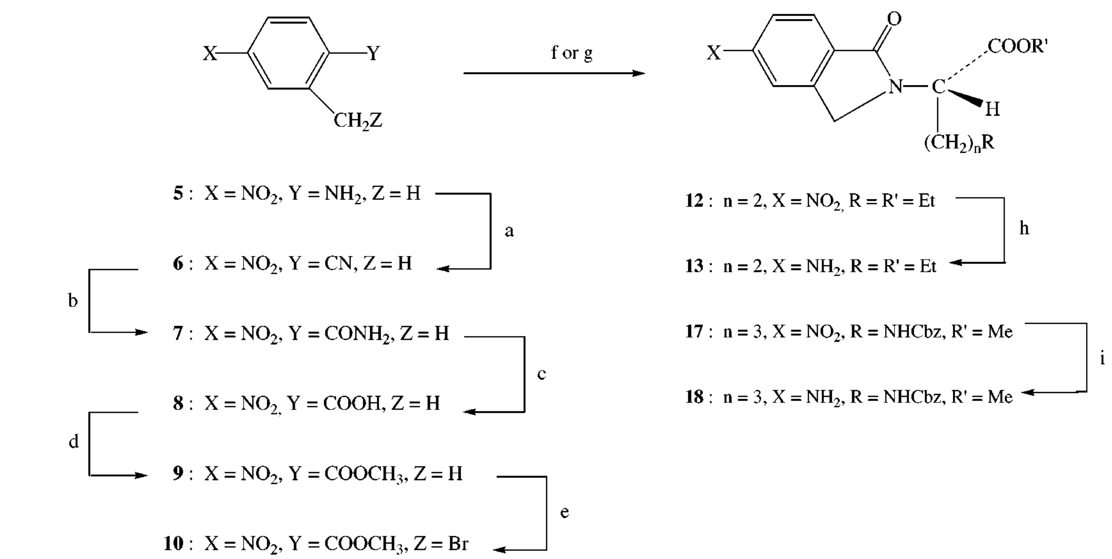
2: R = COOH

4: R = CH_2NH_2

Chemistry

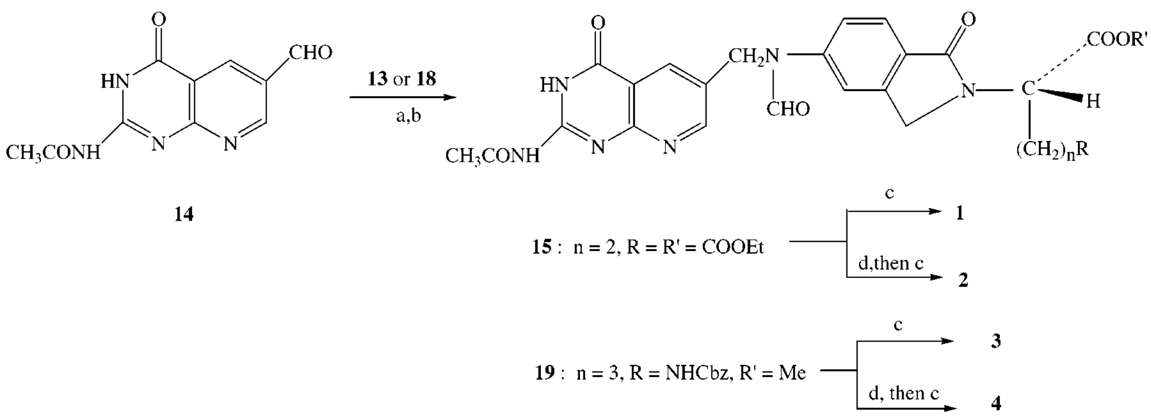
The synthesis of compounds **1-4** is depicted in Scheme 1 and began with commercially available 2-methyl-4-nitroaniline (**5**), which was converted in four steps to the key intermediate methyl 2-bromomethyl-4-nitrobenzoate (**10**) via the nitrile **6**, the amide **7**, the acid **8**, and the ester **9**. The preparation of **6**−**9** was originally described in a publication which is not widely available,[39] whereas a more recent paper[36] describing the use of these compounds as intermediates for the synthesis of rotationally restricted folate antagonists only referred to the earlier work but gave no synthetic details. The

Scheme 1[a]



[a] Reagents: (a) HNO_2/CuCN + KCN; (b) 80% H_2SO_4; (c) 6 N HCl; (d) $SOCl_2$/MeOH; (e) NBS/Bz_2O_2; (f) $H_2NCH(COOEt)CH_2CH_2COOEt$ (**11**)/K_2CO_3; (g) $H_2NCH(COOMe)CH_2CH_2NHCbz$/$K_2CO_3$ (**16**); (h) H_2/5% Pd−C; (i) $SnCl_2$.

Scheme 2[a]



[a] Reagents: (a) $Me_2NH{\cdot}BH_3$/AcOH; (b) 5:1 HCOOH/Ac_2O; (c) HBr/AcOH; (d) H_2/PtO_2/TFA.

Sandmeyer-type reaction of **5** with CuCN afforded **6** in 81% yield, but only when an excess of KCN was added in order to solubilize the CuCN. Conversion of **6** to **7** was accomplished in 98% yield by heating with 80% H_2SO_4 at 100 °C. An attempt to carry out this reaction with alkaline H_2O_2 was unsuccessful. Hydrolysis of the amide group to form **8** was accomplished in 88% yield by heating in 6 N HCl under reflux. Unlike the reaction of **7**, direct hydrolysis of nitrile **6** to acid **8** with 6 N HCl proceeded in low yield because of the facile tendency of the nitrile to steam distill into, and block, the reflux condenser. Esterification of **8** with $SOCl_2$/MeOH yielded **9** (99% yield), and radical bromination of the 2-methyl group with *N*-bromosuccinimide and a catalytic amount of benzoyl peroxide yielded the bromide **10**.[36] Separation of **10** from unreacted **9** was difficult, and we therefore used the oily mixture (93% yield, ca. 70% purity by ^1H NMR) directly in the next step. Treatment with excess diethyl L-glutamate hydrochloride (**11**·HCl) and K_2CO_3 in DMF at room temperature for 3 days afforded isoindoline **12** in 51% yield as waxy solid. Reduction of the nitro group in **12** (H_2/5% Pd−C) yielded the amine **13** (92%) as an oil. Further reaction of **13** with 2-acetamido-6-formylpyrido[2,3-*d*]pyrimidin-4(3*H*)-one (**14**)[16b] and Me_2NH-BH_3 in glacial AcOH at room temperature for 20 h (Scheme 2), followed by treatment with a 5:1 mixture of 95% formic acid and acetic anhydride for 1.5 h at room temperature, afforded a complex mixture from which **15** could be isolated as a pale-yellow solid (37%) by chromatography on silica gel. Simultaneous removal of the ester and amide blocking groups by heating with HBr in AcOH at 80 °C for 15 min then gave **1**, isolated as a colorless solid (68%) by preparative HPLC on C_{18} silica gel followed by ion-exchange chromatography on DEAE-cellulose (HCO_3^- form). To obtain the tetrahydro analogue **2**, the protected intermediate **15** was subjected to catalytic hydrogenation in TFA solution, and the crude reduced product was heated directly with HBr in AcOH (70 °C, 15 min). Preparative HPLC followed by ion-exchange chromatography on DEAE-cellulose afforded **2** as a colorless solid in 30% yield. Formylation of N10 serves a useful function in this sequence by preventing unwanted reductive cleavage of the C9−N10 bond during the hydrogenation step.[16b]

For the synthesis of the ornithine analogue **3**, bromide **10** was treated with excess methyl N^{δ}-Cbz-L-ornithinate (**16**)[24c] in the presence of K_2CO_3 in DMF solution at room temperature for 4 days to obtain the isoindolinone **17** (72%). Since catalytic hydrogenation of the nitro group was precluded by the presence of a Cbz group, reduction

Table 1. Binding of Rotationally Restricted 5-Deaza-5,6,7,8-tetrahydrofolate Analogues to Recombinant Human FPGS[a]

K_m or K_i (μM)	1	2	3	4	22
K_m	2.3 ± 0.6	0.018 ± 0.007	–	–	0.26 ± 0.10
K_i	–	–	110 ± 43	0.20 ± 0.07	–

[a] K_m values for **1**, **2**, and **22** as competitive substrates were determined in the presence of 10 μM (6*S*)-tetrahydrofolate, whereas K_i values for **3** and **4** as competitive inhibitors were determined in the presence of 50 gmM aminopterin. Assays were performed as described in ref 19. Each value is the mean ± standard deviation for three separate experiments done on different days.

was carried out with $SnCl_2$ to form the amine **18** in 75% yield after chromatography on silica gel. Reductive coupling of **18** with aldehyde **14** (Scheme 2), followed by N10-formylation with 5:1 HCOOH−Ac_2O and chromatographic purification on silica gel, yielded the protected intermediate **19** (43%). A faster-moving impurity was also isolated, which was formulated as the *N*-formyl derivative of **18** on the basis that treatment with HCl in MeOH converted it back to **18**. Deprotection of **19** with HBr/AcOH gave a 35% yield of **3** as a pale-yellow powder after HPLC purification. Catalytic hydrogenation in TFA and removal of the blocking groups with HBr in AcOH converted **19** to **4** in 50% yield after ion-exchange chromatography followed by preparative HPLC.

Interaction with FPGS

Compounds **1** and **2** were tested as FPGS substrates, and compounds **3** and **4** were tested as FPGS inhibitors according to a previously described assay which uses aminopterin and [^{3}H]glutamic acid as the cosubstrates.[40] The assay depends on the formation of a charcoal-adsorbable radioactive product that can be readily separated from unreacted [^{3}H]glutamic acid. Human cytosolic recombinant FPGS cloned from human leukemic cells, expressed in insect cells, and purified to homogeneity was used as the enzyme.[41,42] Because of their exceptionally high efficiency as substrates, the K_m values of **1** and **2** were determined by an alternate assay method[20,43] in which (6*S*)-5,6,7,8-tetrahydrofolate is used as the competing substrate and the product is converted to a 5,10-methylene derivative with formaldehyde, allowing it to be trapped as a covalent complex with 5-fluoro-2′-deoxyuridylate (FdUMP) and thymidylate synthase.[44]

As shown in Table 1, the bridged ornithine analogues **3** and **4** were both inhibitors of FPGS, with K_i values of 110 and 0.20 μM, respectively. The >100-fold difference in K_i between these two compounds was consistent with our previous results on 5-deazapteroyl-L-ornithine (**20**) versus its (6*R*,6*S*)-tetrahydro derivative **21**, which gave K_i values against mouse FPGS of 5.7 and 0.030 μM, respectively.[24d] While the weaker binding we observed for the bridged relative to the nonbridged analogues could be due to the different species of origin of the two enzymes, it is known from other studies that the K_i values of ornithine-containing inhibitors of mouse and human FPGS are not very different; in the case of *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-ornithine, for example, these values are reported to be 20.4 μM[24a] and 13.4 μM,[24b] a difference of less than 2-fold. Thus we believe that the lower inhibitory activity of **4** versus **20** and **3** versus **21** is due to the bridge between the phenyl ring and the amide nitrogen.

When the glutamate-containing analogues **1** and **2** were tested as substrates, their K_m values were found to be 2.3 and 0.018 μM. In contrast, (6*R*,6*S*)-5-deazatetrahydrofolate (**22**) gave a K_m of 0.26 μM. Thus, rotational restriction of the amide bond in **2** appeared to cause a 14-fold increase in binding to the human enzyme. It is of interest that the K_m values reported earlier for **22** and its nonreduced analogue **24** as substrates for murine FPGS were 9.7 and 157 μM, respectively.[16a] To our knowledge, **2** is the best FPGS substrate described to date. Indeed, estimation of its K_m required us to employ a different assay method than the one typically used with most known FPGS substrates. The V_{max} for the FPGS-catalyzed reaction of **2** was roughly equivalent to that of aminopterin (data not shown).[45]

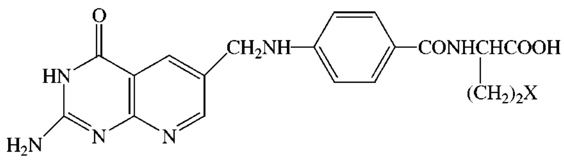
The major natural tetrahydrofolate cofactors are all reported to have K_m values in the micromolar range as substrates for both murine FPGS (10-formyltetrahydrofolate, 3.9 μM; 5,10-methylenetetrahydrofolate, 4.8 μM; tetrahydrofolate, 7.0 μM; 5-methyltetrahydrofolate, 87 μM)[40] and human FPGS (10-formyltetrahydrofolate, 3.7 μM; tetrahydrofolate, 4.4 μM; 5-methyltetrahydrofolate, 48 μM).[42b] Several antifolates are also reported to have K_m values in the micromolar range for this enzyme. For example, the potent thymidylate synthase inhibitor tomudex (ZD1694) has a K_m of 1.37 μM, while the glycinamide ribonucleotide formyltransferase inhibitor (6*R*)-5,10-dideazatetrahydrofolate (lometrexol, **23**) and the multitargeted antifolate LY231514 (**25**), which are conformationally unrestrained in the side chain, have K_m values of 9.3 and 0.8 μM, respectively.[16e] Another excellent FPGS substrate is the tricyclic TS inhibitor BW1843U89 (**26**), with a reported K_m of 0.41 μM for the hog liver enzyme.[37a] An important conclusion suggested by our results is that the low K_m of **26** is probably due more to the conformational rigidity of the bridged amide bond than to the tricyclic nature of the benzo[*f*]quinazoline moiety.

A significant feature of **26** is that, despite its low substrate K_m for FPGS, catalysis is efficient only for the addition of the first glutamyl residue. Thus, in contrast to nonbridged analogues such as **23**[47] and **25**,[48] the predominant metabolite of **26** is the diglutamate.[37c] It was therefore of interest to determine whether the amide bridge in **2** similarly causes the FPGS reaction to become arrested at the first step. An experiment was carried out in which the distribution of radioactive metabolites was examined by HPLC after incubating **1**, **2**, and **22** with [^{3}H]L-glutamic acid and human FPGS for 10 min and 2 h. As shown in Table 2, conversion of **22** to its long-chain polyglutamates was highly efficient, so that by 2 h the only radioactive species detected by HPLC were the penta- and hexaglutamate compounds. In sharp contrast, the reaction of both **1** and **2** was arrested after the addition of one glutamic acid residue, although there was evidence for very slight conversion of **2** to longer conjugates. Thus, the behavior of these rotationally restricted analogues was similar to that of

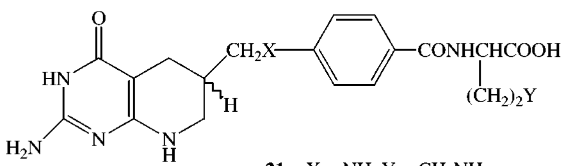
Table 2. Effect of Rotational Restriction on the Polyglutamate Chain Length of 5-Deazatetrahydrofolate Analogues[a]

		number of glutamates added					
compd	time (min)	0	1	2	3	4	5
1	10	13.4	86.6	0	0	0	0
	120	15.0	83.9	2.0	0	0	0
2	10	6.9	93.1	0	0	0	0
	120	0	95.5	1.3	1.7	1.5	0
22	10	10	7.8	46.7	32.7	4.0	0
	120	0	0	0	0	67.4	32.6

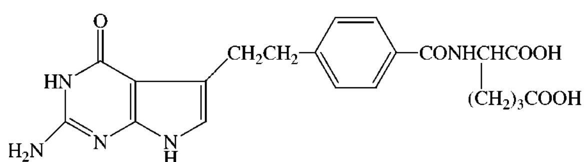
[a] Compounds **1** (5 μM), **2** (3.5 μM), andr **22** (5 μM) were incubated for 10 or 120 min with 1 μg of recombinant human cytosolic FPGS in 200 mM Tris buffer, pH 7.9, with 10 mM $MgCl_2$, 5 mM ATP, 2.5 mM [^{3}H]L-glutamic acid, 20 mM 2-mercaptoethanol, and 30 mM KCl in a total volume of 500 μL. Radioactive polyglutamates were separated by paired-ion reverse-phase HPLC as described in ref 52. The chain length of the products was determined by the ratio of ^{3}H to UV absorbance, normalized with respect to the ratio for the diglutamate.



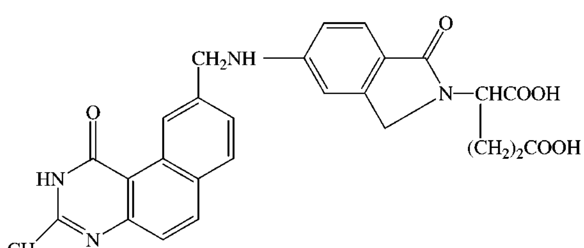
20 : X = CH_2NH_2
24 : X = COOH



21 : X = NH, Y = CH_2NH_2
22 : X = NH, Y = COOH
23 : X = CH_2, Y = COOH



25 (LY231514)



26 (BW1843)

26, in that they were rapidly metabolized by FPGS but were only converted in significant amount to the diglutamate.

A hypothetical model for the binding of folate and antifolates substrates to FPGS was recently proposed on the basis of a crystallographic analysis of recombinant enzyme cloned from *Lactobacillus casei*.[49] Although the structure was only solved for the ATP−protein complex (i.e. without a folyl ligand), it was found that the enzyme consists of two distinct domains, one of which resembles the folate binding region of DHFR, whereas the other has the typical features of an ATP binding motif. Not surprisingly in view of the demonstrated role of ATP in activating the γ-carboxyl group during the FPGS reaction,[50] the glutamate tail of the folyl substrate is believed to extend across the interdomain cleft and into the ATP binding site. While the three-dimensional structure of the mammalian enzyme has yet to be solved, the high sequence homology between human and *L. casei* FPGS suggests that the two proteins will prove to have similar structures. Thus the effects of conformational restriction on the binding of folyl derivatives may be due to differences in the ease with which the γ-carboxyl group of the side chain can extend into the ATP binding domain. A better understanding of how rotational restriction about the amide bond affects the binding of substrates such as **1** and **2** and inhibitors such as **3** and **4** to FPGS must await three-dimensional structural analysis of crystalline ternary complexes of the enzyme with ATP and a folyl ligand.

Cell Growth Inhibition

Assays of **1**−**4** as inhibitors of the growth of cultured CCRF-CEM cells during 72 h of drug exposure showed these compounds to be inactive at concentrations of up to 100 μM (data not shown). The reported IC_{50} values of the nonbridged glutamate **22** against CCRF-CEM cells is reported to be 0.01 μM,[16b] and that of the nonreduced analogue **26** (against L1210 cells) is reported to be 8.8 μM.[51] Thus, except for the important information provided by bridged compounds **1**−**4** with regard to the binding preferences of FPGS ligands, further assessment of the interaction of these weakly cytotoxic compounds with other enzymes of folate metabolism, such as GARFT, TS, or DHFR, was not considered worthwhile.

Experimental Section

UV spectra were obtained on a Varian model 210 instrument, and ^{1}H NMR spectra were obtained with a Varian EM360L instrument using Me_4Si as the reference. IR spectra (data omitted for brevity) were routinely obtained on all compounds and were consistent with assigned structures. TLC analyses were done on fluorescent Whatman MKSF silica gel-coated glass slides (60 Å layer), using 254-nm UV illumination to visualize spots. Column chromatography was on Baker 3405 (60−200 mesh) silica gel or Whatman DE-52 preswollen DEAE-cellulose (HCO_3^- form). Solvents in moisture-sensitive reactions were of Sure-Seal grade (Aldrich, Milwaukee, WI) or were dried over Linde 4A molecular sieves (Fisher, Boston, MA). HPLC purification of the final products was on Waters C_{18} radial compression cartridges (analytical: 5-μm particles, 5 × 100 mm; preparative: 15-μm particles, 25 × 100 mm). In those instances where HPLC was followed by ion-exchange chromatography, the latter was performed in order to ensure that the sample was not contaminated with silica gel, which can occur from C_{18} columns on repeated use. Diethyl 2-L-[*N*-(2,3-dihydro-5-nitro-1-oxo-2(1*H*)-isoindolyl)amino]glutatarate (**12**),[36] 2-acetamido-6-formylpyrido[2,3-*d*]pyrimidin-4(3*H*)-one (**14**),[16b] and methyl N^{δ}-Cbz-L-ornithinate (**16**)[24c,d] were synthesized according to published methods. A minor modification of the synthesis of **12** was that 1,2-dichloroethane was used instead of carbon tetrachloride as the solvent for the radical bromination of **9**. Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were performed by Quantitative Technologies, Inc., Whitehouse, NJ, and were within ±0.4% of calculated values unless otherwise indicated.

Aminopterin and other chemicals for the FPGS assays were purchased from Sigma (St. Louis, MO). [3,4-^{3}H]L-Glutamic acid was obtained from DuPont-New England Nuclear (Wilmington, DE). The scintillation cocktail was Safety-Solve from Research Products International (Mount Prospect, IL). (6*S*)-Tetrahydrofolate was prepared enzymatically.[44] *Lactobacillus casei* thymidylate synthase was expressed in an *Escherichia coli* transfectant kindly provided by Dr. Daniel Santi (University of California, San Francisco) and was purified by phosphocellulose chromatography. Cloning, expression, and purification to electrophoretic homogeneity of FPGS from CCRF-CEM human leukemic lymphoblasts were carried out as reported earlier.[41]

Methyl 2-Methyl-4-nitrobenzoate (9). Crushed ice (100 g) was added to a suspension of 2-methyl-4-nitroaniline (**5**) (15.2 g, 0.1 mol) in 12 N HCl (25 mL) and the mixture was stirred at 0−5 °C while adding a solution of $NaNO_2$ (7.0 g, 0.1 mol) in H_2O (20 mL) over a period of 15 min. Despite the addition of an extra 10% of the $NaNO_2$ solution, some starting material remained undissolved. After careful neutralization at 0−5 °C with aqueous Na_2CO_3 (**caution**: CO_2 evolution), the neutralized mixture was added dropwise with cooling and stirring to a two-phase mixture of EtOAc (100 mL) and a solution of CuCN (8.95 g, 0.1 mol) and KCN (15 g, 0.23 mol) in H_2O (50 mL). After addition was complete the mixture was allowed to warm to room temperature and left to stand overnight. The mixture was filtered, the organic layer was separated, the aqueous layer was extracted with EtOAc, and the combined EtOAc layers were evaporated. The residue was stirred in warm hexane, enough acetone was added to dissolve almost all the solid, the warm mixture was filtered, and the filtrate was cooled until crystals of nitrile **6** were obtained: yield 13.2 g (81%); mp 99−100 °C; ^{1}H NMR ($CDCl_3$) δ 2.7 (s, 3H, Me), 7.8 (d, J = 10 Hz, 1H, 6-H), 8.2 (s, 1H, overlapping dd, 1H, 3-H and 5-H).

A solution of **6** (11.5 g, 0.0707 mol) in 80% H_2SO_4 (75 mL) was heated in an oil bath at 100 °C for 1.25 h, then cooled, and poured onto ice. The precipitate was collected and dried on a lyophilizer to obtain amide **7** as a light-yellow solid (12.5 g, 98%): mp 167−168 °C (lit.[39] mp 167 °C); ^{1}H NMR ($CDCl_3$) δ 2.6 (s, 3H, Me), 7.6 (d, J = 8 Hz, 1H, 5-H), 8.1 (m, 2H, 3-H and 6-H).

A stirred mixture of **7** (12.5 g, 0.0693 mol) and 6 N HCl (500 mL) was refluxed for 20 h, and the black solid which remained undissolved was removed by filtration and repeatedly extracted with hot water until no more of it dissolved. Cooling of the combined filtrate and extracts caused precipitation of a solid, which was filtered, washed with H_2O, and dried on a lyophilizer to obtain acid **8** as a yellow solid: yield 11.0 g (88%); mp 154−155 °C (lit.[39] mp 153−154 °C); ^{1}H NMR (DMSO-d_6) δ 2.6 (s, 3H, Me), 8.1 (m, 3H, aryl).

Thionyl chloride (18 g, 11 mL, 0.15 mol) was added dropwise to a stirred solution of **8** (12.4 g, 0.0683 mol) in MeOH (100 mL) while maintaining the internal temperature below 12 °C. When addition was complete the mixture was left to stand at room temperature for 21 h to obtain needles of **9** (6.80 g): mp 74 °C (lit.[39] mp 68−69 °C). Evaporation of the filtrate yielded another 6.35 g of solid with mp 68−69 °C: total yield 13.2 g (99%); ^{1}H NMR ($CDCl_3$) δ 2.7 (s, 3H, Me), 4.0 (s, 3H, OMe), 8.1 (m, 3H, aryl).

Diethyl 2-L-[5-[*N*-(2-Acetamido-4(3*H*)-oxopyrido[2,3-*d*]-pyrimidin-6-yl)methylformamido]-1-oxo-2(1*H*)-isoindolyl]-glutarate (15). A solution of **9** (6.35 g, 0.0326 mol) in 1,2-dichloroethane (50 mL) was treated with *N*-bromosuccinimide (5.80 g, 0.0326 mol) and benzoyl peroxide (50 mg), and the mixture was refluxed for 2 days, then cooled, washed with H_2O, and evaporated to an oil (8.32 g, 93%): ^{1}H NMR ($CDCl_3$) δ 2.68 (s, ca. 0.9H, 2-CH_3 of **9**), 4.02 (s, 3H, OCH_3 in **9** + **10**), 4.97 (s, ca. 1.4H, CH_2Br), 8.18 (m, 3H, aromatic protons in **9** + **10**). The product was estimated from the ^{1}H NMR spectrum to be a 7:3 mixture of **10** and unreacted **9**. Direct reaction of this mixture with diethyl L-glutamate[36] afforded nitroindolinone **12** in 51% yield based on the estimated amount of **10** in the reaction: mp 78−79 °C (lit.[36] mp 73−74 °C). Catalytic hydrogenation of **12** over 5% Pd−C as described[36] then gave the amino isoindolinone **13** as an oil (0.84 g, 92%): ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 6H, two CH_2CH_3), 2.3 (m, 4H, two glutaryl CH_2), 4.1−4.6 (complex m, 6H, two CH_2CH_3 and isoindolinyl CH_2), 5.0 (m, 1H, glutaryl CH), 6.7 (m, 2H, isoindolinyl 4-H and 6-H), 7.7 (d, J = 8 Hz, isoindolinyl 7-H).

A solution of **13** (344 mg, 1.03 mmol) and **14** (232 mg, 1.00 mmol) in glacial AcOH (5 mL) was stirred at room temperature for 20 h, then treated with $Me_2NH{\cdot}BH_3$ (41 mg, 0.70 mmol). Stirring was continued at room temperature for 1 h and at 60−65 °C (internal) for 10 min. The solvent was evaporated and the residue dissolved in a ice-cold mixture of Ac_2O (1 mL) and 95% HCOOH (5 mL) which had been made up in advance. The reaction mixture was kept at room temperature for 1.5 h before being evaporated under reduced pressure. Analysis by TLC (silica gel, 2:1 acetone−hexane) showed a spot at the origin and three mobile spots with R_f values of 0.1 (coupling product **15**), 0.3, and 0.6. With 10:1 $CHCl_3$−MeOH, the R_f of **15** was 0.5. The crude mixture was chromatographed twice on silica gel (20 g, 2 × 14 cm), first with 2:1 acetone−hexane as the eluent and then with 20:1 acetone−MeOH. Pooled TLC homogeneous fractions were evaporated and the residue was dried in vacuo at 70 °C over P_2O_5 to obtain a pale-yellow solid (215 mg, 37%): mp 118−121 °C; ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 6H two CH_2CH_3), 2.4 (m, 4H, β- and γ-CH_2), 2.5 (s, 3H, CH_3-CO), 4.1−4.6 (complex m, 6H, two CH_2CH_3, isoindolinyl CH_2), 5.2 (m, 3H, 9-CH_2 and α-CH), 7.2 (m, 2H, isoindolinyl 4-H and 6-H), 7.9 (d, J = 8 Hz, 1H, isoindolinyl 7-H), 8.4 (d, J = 2 Hz, 1H, pyridyl 5-H), 8.7 (s, 1H, CH=O), 8.93 (d, J = 2 Hz, 1H, pyridyl 7-H). Anal. ($C_{28}H_{30}N_6O_8{\cdot}0.5H_2O$) C, H, N.

2-L-[5-[*N*-(2-Amino-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methylamino]-1-oxo-2(1*H*)-isoindolyl]-L-glutaric Acid (1). A solution of **15** (134 mg, 0.232 mmol) in glacial AcOH (4 mL) was treated with 48% aqueous HBr (4 mL) and heated to 80 °C (internal) for 15 min. After rotary evaporation the residue was taken up in dilute ammonia and purified by preparative HPLC (C_{18} silica gel, 4% MeCN in 0.1 M NH_4OAc, pH 6.9, 1.0 mL/min, 280 nm) followed by ion-exchange chromatography on DEAE-cellulose (HCO_3^- form) using 0.4 M NH_4HCO_3 as the initial eluent, then 0.4 M NH_4HCO_3 adjusted to pH 10 with 28% NH_4OH. Pooled pure fractions were lyophilized to dryness to obtain a white powder (80 mg, 68%): mp >240 °C dec; UV (0.1 N NaOH) λ_{max} 219 nm (ϵ 30 200), 240 infl (24 600), 297 (20 400), 280 infl (19 400); ^{1}H NMR (DMSO-d_6 + 1 drop each of TFA and D_2O) δ 2.2 (m, 4H, β- and γ-CH_2), 4.2−4.9 (m, 5H, 9-CH_2, α-CH, and indolinyl CH_2), 6.8 (m, 2H isoindolinyl 4-H and 6-H), 7.4 (d, J = 9 Hz, 1H, isoindolinyl 7-H), 8.7 (m, 2H, pyridyl 5-H and 7-H). Anal. ($C_{21}H_{20}N_6O_8{\cdot}3H_2O$) C, H, N.

2-L-[2,3-Dihydro-5-[*N*-[(6*R*,6*S*)−2-amino-4(3*H*)-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl]methylamino]-1-oxo-2(1*H*)-isoindolyl]glutaric Acid (2). A solution of **15** (109 mg, 0.188 mmol) in TFA (8 mL) in a Parr apparatus was shaken with PtO_2 (15 mg) under H_2 (3 atm) for 1 h. The catalyst was filtered and washed with glacial AcOH, and the combined filtrate and washings were evaporated under reduced pressure. The residue was redissolved in a glacial AcOH (4 mL) and 48% aqueous HBr (4 mL) and the mixture was heated at 70 °C (internal) for 15 min. The solution was re-evaporated to dryness, and the residue was stirred in H_2O while enough 28% NH_4OH was added to dissolve nearly all of the solid. The insoluble portion was removed and the product was purified by preparative HPLC followed by ion-exchange chromatography as described in the preceding experiment. Lyophilization of appropriately combined fractions from the ion-exchange column yielded **2** as a white powder (49 mg, 30%) whose elemental analysis indicated that it was a partial ammonium salt: mp >240 °C dec; UV (0.1 N NaOH) λ_{max} 279 nm (ϵ 24 100), 292−302 (21 500); ^{1}H NMR (DMSO-d_6 + 1 drop of D_2O) δ 2.2 (m, 5H, β- and γ-CH_2, tetrahydropyridyl 6-CH), 3.1 (m, 4H, 7- and 9-CH_2), 4.4 (m, 3H, isoindolinyl CH_2 and glutaric acid α-CH), 6.7 (m, 2H, isoindolinyl 4-H and 6-H), 7.4 (1H, d, J = 8 Hz, isoindolinyl 7-H). The tetrahydropyridyl 4-CH_2 group, assumed to be at δ 3.6, was obscured by a large H_2O peak. Anal. ($C_{21}H_{24}N_6O_6$·0.5NH_3·2H_2O) C, H, N.

Methyl 2-L-(2,3-Dihydro-5-nitro-1-oxo-2(1*H*)-isoindolyl)-5-[*N*-(benzyloxycarbonyl)amino]pentanoate (17). A stirred solution of bromide **10** (6.24 g, 0.0228 mol, based on ^{1}H NMR) in dry DMF (50 mL) was treated with **16** (7.22 g, 0.0228 mol) and K_2CO_3 (6.9 g, 50 mmol), and after 4 days the mixture was diluted with EtOAc and filtered. The filter cake was washed with EtOAc, and the combined filtrate and washings were rinsed with H_2O. TLC (silica gel, 2:1 hexane−acetone) of the organic layer showed **17** as a strong spot with R_f 0.3. The organic layer was evaporated onto silica gel, and the dried silica gel was applied to the top of a column of flash-grade silica gel (50 g, 4 × 9 cm), which was eluted successively with 2:1 hexane−acetone and then with 3:2 hexane−acetone. Evaporation of appropriately combined eluates yielded a yellow solid (7.24 g, 72%): mp 83−85 °C. Recrystallization from a mixture of hexane and acetone afforded the analytical sample as an off-white solid: mp 95−96 °C with prior softening; ^{1}H NMR ($CDCl_3$) δ 1.6 (m, 4H, pentanoic acid β- and γ-CH_2), 3.3 (q, J = 6 Hz, 2H, CH_2NH), 3.7 (s, 3H, OCH_3), 4.5−5.0 (m, 3H, pentanoic acid α-CH, isoindolinyl CH_2), 5.1 (s, 2H, benzylic CH_2), 7.3 (s, 5H, phenyl), 8.0 (d, J = 10 Hz, 1H, isoindolinyl 6-H), 8.3 (m, 2H, isoindolinyl 4-H and 7-H). Anal. ($C_{22}H_{23}N_3O_7$) C, H, N.

Methyl 2-L-(5-Amino-2,3-dihydro-1-oxo-2(1*H*)-isoindolyl)-5-[*N*-(benzyloxycarbonyl)amino]pentanoate (18). A solution of the nitro isoindolinone **17** (0.93 g, 0.0211 mol) in EtOAc (25 mL) was treated with $SnCl_2$·2H_2O (2.37 g, 0.0105 mol), and after being heated to reflux for 1.5 h the mixture was cooled and quenched with 5% aqueous $NaHCO_3$. The inorganic salts were filtered, the two layers of the filtrate were separated, and the EtOAc layer was evaporated to a foam (0.70 g, 80% crude yield): R_f 0.4 (silica gel, EtOAc). Column chromatography on silica gel (15 g, 2 × 11 cm) with EtOAc as the eluent gave pure **18** as a hardened straw-colored foam (0.65 g, 75%): mp 49−52 °C; ^{1}H NMR ($CDCl_3$) δ 1.20 (m, >4H, pentanoyl β- and γ-CH_2 overlapping residual H_2O in the sample), 3.7 (s, 3H, OCH_3), 3.9−4.4 (m, 4H, NH_2, isoindolinyl CH_2), 5.0 (m, 4H, NH, benzylic CH_2, pentanoic acid α-CH), 6.6 (m, 2H, isoindolinyl 4-H and 6-H), 7.3 (s, 5H, phenyl), 7.7 (d, J = 7 Hz, 1H, isoindolinyl 7-H). Anal. ($C_{22}H_{25}N_3O_5$·0.5H_2O) C, H, N.

2-L-[5-[*N*-(2-Amino-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methylamino]-2,3-dihydro-1-oxo-2(1*H*)-isoindolyl]-5-aminopentanoic Acid (3). A mixture of **14** (360 mg, 155 mmol) and the aminoisoindolinone **18** (630 mg, 153 mmol) in glacial AcOH (10 mL) was stirred at room temperature for 1 day before being treated with Me_2NH·BH_3 (59 mg, 1.00 mmol). Stirring was continued at room temperature for 1 h and then at 60−65 °C (internal) for 10 min. The solvent was evaporated and the residue dissolved in 95% HCOOH, cooled in an ice bath, and treated with premixed ice-cold 95% HCOOH (5 mL) and Ac_2O (1 mL). After 1.5 h at room temperature the solution was evaporated, and the residue was redissolved in a mixture of acetone and MeOH and evaporated onto silica gel (2 g). The dried silica gel was placed on top of a silica gel column (20 g, 2 × 15 cm) which was eluted first with 2:1 acetone−hexane to remove a fast moving impurity with an R_f value of 0.4 (silica gel, 2:1 acetone−hexane), which appeared to be the N-formyl derivative of **18** (it was converted back to **18** upon reaction with HCl in MeOH). After this impurity was removed the column was eluted with 20:1 acetone−MeOH to recover a solid (534 mg) whose TLC (silica gel, 10:1 $CHCl_3$−MeOH) contained a major spot with R_f 0.5 (coupling product **19**) and a minor contaminant with R_f 0.3. Rechromatography using the same system, with acetone being used to rinse collection tubes from the column, yielded **19** as a hardened straw-colored foam (489 mg, 43%) which was pure enough to be used in the next step: softening 125−130 °C; ^{1}H NMR ($CHCl_3$) δ 1.3−2.4 (m, >4H, β- and γ-CH_2, CH_3 from residual acetone in the sample), 2.5 (s, 3H, CH_3CO), 3.3 (m, 2H, δ-CH_2), 3.7 (s, 3H, CH_3), 4.5 (d, J = 7 Hz, isoindolinyl CH_2), 5.2 (m, 5H, 9-CH_2, benzylic CH_2, and pentanoic acid α-CH), 7.4 (s, 7H, phenyl, isoindolinyl 4-H and 6-H), 7.9 (d, J = 10 Hz, 1H, isoindolinyl 7-H), 8.5 (d, J = 2 Hz, 1H, pyridyl 5-H), 8.7 (s, 1H, CH=O), 8.9 (d, J = 2 Hz, 1H, pyridyl 7-H).

The protected derivative **19** (187 mg, 0.285 mmol) was dissolved in 6 mL of 15% HBr in AcOH with the aid of an ultrasonic bath, kept at room temperature for 30 min, treated with 16% aqueous HBr (3 mL), heated at 70 °C (internal) for 15 min, and evaporated to dryness. Analytical HPLC (C_{18} silica gel, 1% AcOH, 5% EtOH, 1 mL/min) showed a major peak at 9.0 min corresponding to the product **3**, along with minor peaks at 4.0, 6.2, and 19.0 min. The solid was stirred in H_2O while adding NaOH dropwise until a clear solution formed (pH >10). The solution was placed on top of a column of Dowex 50W-X2 (H^+ form, 2 × 21 cm) which was eluted first with distilled H_2O until the eluate was neutral (desalting step) and then with 3% NH_4OH. Fractions were monitored by analytical HPLC for the appearance of a peak eluting at 9.0 min. Appropriate fractions were pooled, reduced to a smaller volume, filtered through a sintered glass filter to remove some turbidity, and finally purified by preparative HPLC (C_{18} silica gel, 1% AcOH, 5% EtOH). The product eluting at 9.0 min was collected and lyophilized, the resulting amorphous solid taken up in dilute ammonia, and the solution relyophilized to obtain a pale-yellow powder (49 mg, 35%): mp >240 °C; ^{1}H NMR (DMSO-d_6 + 2 drops TFA) δ 1.3−2.3 (m, 4H, β- and γ-CH_2), 2.6−3.0 (m, 2H, δ-CH_2), 4.1−4.9 (complex m, 5H, α-CH, 9-CH_2, and isoindolinyl CH_2), 6.8 (m, 2H, 2-NH_2), 7.3−8.0 (m, 6H, δ-NH_3+, isoindolinyl 4-H, 6-H, and 7-H), 8.4 (broad m, 1H, NH), 8.6 (d, J = 2 Hz, 1H, pyridyl 5-H), 8.7 (d, J = 2 Hz, 1H, pyridyl 7-H). Anal. ($C_{21}H_{23}N_7O_4$·3H_2O) C, H, N.

2-L-[5-[*N*-[(6*R*,6*S*)-2-Amino-4(3*H*)-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl]methylamino]-2,3-dihydro-1-oxo-2(1*H*)-isoindolyl]-5-aminopentanoic Acid (4). A solution of **19** (210 mg, 0.321 mmol) in TFA (8 mL) was shaken in a Parr apparatus with PtO_2 (20 mg) under 3 atm of H_2 for 1 h. The catalyst was filtered and washed with glacial AcOH, the combined filtrate and washings were evaporated, the residue was taken up in a mixture of glacial AcOH (4 mL) and 48% aqueous HBr (4 mL), and the solution was heated at 80 °C (internal) for 15 min. After evaporation of the solvent the residue was dissolved in H_2O by addition of enough NaOH to raise the pH above 10. A small amount of insoluble material was removed by filtration; the filtrate was added to the top of an ion-exchange column (Dowex 50W-X2, H^+ form, 2 × 25 cm). The column was eluted with distilled H_2O until the pH of the eluate was neutral (desalting step) and then with 3% NH_4OH while monitoring fractions by HPLC (C_{18} silica gel, 1% AcOH, 5% EtOH, 1.0 mL/min). Fractions showing a peak at 12 min were pooled, concentrated to ca. 70 mL, treated with glacial AcOH (0.7 mL), and chilled. A very small amount of solid which precipitated was collected, the filtrate was repurified by HPLC, and the pooled eluates showing a single peak at 12 min were freeze-dried to an off-white solid (90 mg, 50%): mp >240 °C; ^{1}H NMR (DMSO-d_6 + 1 drop TFA) δ 1.4−3.5 (complex m, 16H, *C*H_3COOH, β-, γ-, and δ-CH_2, 9-CH_2, and tetrahydropyridyl 5-CH_2, 6-CH, and 7-CH_2), 4.4 (m, 2H, isoindolinyl CH_2), 4.8 (m, 1H, pentanoic acid α-CH), 6.3 (s, 1H, NH), 6.8 (m, 2H, 2-NH_2), 7.1 (s, 1H, isoindolinyl 4-H), 7.3 (m, 6H, isoindolinyl 6-H and 7-H, δ-NH_3^+, NH). Anal. ($C_{21}H_{27}N_7O_4$·CH_3COOH·2H_2O) C, H, N.

FPGS Assays. The activity of **1**, **2**, and **22** as FPGS substrates was determined according to a two-stage microassay method[43] which is more satisfactory than the standard charcoal adsorption procedure when the test compound (e.g. **2**) has a low K_m. The practical utility of this coupled assay has been discussed in detail elsewhere.[20] In the first step, different concentrations of the test compound (10−600 μM) were incubated at 37 °C for 30 min in a mixture containing

FPGS, (6*S*)-tetrahydrofolate (10 μM), ATP (5 mM), [^{3}H]L-glutamic acid (1 mM, 4 mCi/mmol), KCl (30 mM), $MgCl_2$ (10 mM), and Tris·HCl, pH 8.5 (200 mM), in a total volume of 10 μL. In the second step, a mixture of purified recombinant *E. coli* TS (1 μM), Na_2HPO_4, pH 7.2 (30 mM), 2-mercaptoethanol (8 mM), formaldehyde (15 mM), and 5-fluoro-2′-deoxyuridylate (2 μM) in a total volume of 100 μL was added, and incubation at 37 °C was continued for another 30 min. The reaction mixture was then passed through a Sephadex G-50 spin column and the eluate analyzed for total ^{3}H by scintillation counting. A Dixon plot was used to estimate the K_m of the competitive substrate from the following equation: *x*-intercept $= -K_m/(1 + [S]/K_{m,tetrahydrofolate})$, where [S] is the substrate concentration. It is assumed in this calculation that the reactions of (6*S*)-tetrahydrofolate and the test compound are mutually exclusive. The K_m of (6*S*)-tetrahydrofolate was taken to be 1.0 μM, based on a separate kinetic determination of this value.

The K_i of **3** and **4** as competitive inhibitors of the FPGS-catalyzed reaction of aminopterin with [^{3}H]L-glutamic acid was determined by the standard charcoal adsorption method as previously described.[13a] Briefly, different concentrations (0−1000 μM) of the test compound were incubated at 37 °C for 1 h in the presence of aminopterin (50 μM), [^{3}H]L-glutamic acid (1 mM, 4 mCi/mmol), ATP (5 mM), $MgCl_2$ (10 mM), KCl, (30 mM), and α-thioglycerol (20 mM) in Tris·HCl, pH 8.6 (200 mM), in a total volume of 0.25 mL. In some experiments the products of the FPGS reaction were separated by paired-ion reverse-phase HPLC.[52]

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