Published in final edited form as:

Arch Biochem Biophys. 2009 August 15; 488(2): 140–145. doi:10.1016/j.abb.2009.06.017.

Inhibition of human folypolyglutamate synthetase by diastereomeric phosphinic acid mimics of the tetrahedral intermediate

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Abstract

Phosphorus-containing pseudopeptides, racemic at the C-terminal α -carbon, are potent mechanism-based inhibitors of folypolyglutamate synthetase (FPGS). They are mimics of the tetrahedral intermediate postulated to form during FPGS-catalyzed biosynthesis of poly(γ -L-glutamates). In the present paper, the FPGS inhibitory activity of each diastereomer coupled to three heterocycles is reported. The high R_f pseudopeptide containing the 5,10-dideazatetrahydropteroyl (DDAH₄Pte) heterocycle is most potent ($K_{is} = 1.7$ nM). While the heterocyclic portion affects absolute FPGS inhibitory potency, the high R_f species is more potent in each pair containing the same heterocycle. This species presumably has the same stereochemistry as the natural folate polyglutamate, i.e., (L-Glu- γ -L-Glu). Unexpectedly, the low R_f (presumed L-Glu- γ -D-Glu) species are only slightly less potent (<30-fold) less potent than their diastereomers. Further study of this phenomenon comparing L-Glu- γ -L-Glu and L-Glu- γ -D-Glu dipeptide-containing FPGS substrates shows that <1% contamination of commercial D-Glu precursors by L-Glu may give misleading information if L-Glu- γ -L-Glu substrates have low K_m values.

Keywords

folypolyglutamate synthetase; phosphapeptide inhibitor; stereospecificity; structure-activity relationship; antifolate; mechanism-based

Introduction

The intracellular folate pool consists of a family of derivatives of folic acid that differ in reduction state, presence and nature of one-carbon substituents, and occurrence of poly(γ -glutamyl) chains ligated to the glutamate (Glu)¹ intrinsic to the folic acid structure [1]. Folates, in their fully reduced 5,6,7,8-tetrahydro state, participate in a variety of anabolic and catabolic one-carbon transfer reactions, including those in thymidylate synthesis, *de novo* purine synthesis, and synthesis of serine, glycine, and methionine [2]. Poly(γ -glutamyl)ation of folates

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with up to seven additional Glu residues in mammalian cells serves two major functions [3]. Polyglutamylation serves to retain folates inside cells since only monoglutamates are substrates for folate efflux systems and the high negative charge associated with polyglutamylation at physiological pH precludes diffusion through the membrane. In addition, polyglutamates serve as the preferred substrates (higher $V_{\max}/K_m$) for virtually all folate-dependent enzymes.

Polyglutamylation of folates is an essential process because mutational deletion of folylpolyglutamate synthetase (FPGS), the sole enzyme responsible for their synthesis, is lethal unless all the end-products of folate metabolism are supplied (i.e., thymidine, purines, serine, glycine, methionine, etc.) [4,5]. This essential requirement for polyglutamylation has led numerous investigators to propose FPGS as a target in cancer chemotherapy [6,7]. Our laboratories have investigated several different classes of potential inhibitors in an effort to identify potent and specific FPGS inhibitors [7–9]. Recently, we described phosphorus-containing pseudopeptides in which the tetrahedral P^V species (as a phosphonate (Figure 1,2a) [10] or a phosphinate (Figure 1,2b) [11,12]) serves as a mimic of the transient tetrahedral intermediate (Figure 1, 1) derived from the γ -glutamyl phosphate reaction intermediate [13]. Within this phosphorus-containing class, phosphinate-based inhibitors are clearly most potent [12]. The phosphinates have another advantage in that there is precedent for further processing of the inhibitor via an ATP-dependent, enzyme-catalyzed reaction to form the phosphorylated phosphinate, **3** [14–18], with significant enhancement of inhibitory activity.

The initial proof-of-concept analogs were synthesized as mixtures of diastereomers [11], because of the lack of synthetic methodology for the stereoselective synthesis of complex phosphinate pseudopeptides. In addition, earlier analogs were all prepared with a single folate like heterocycle (4-amino-4-deoxy-10-methylpteroate; AMPte) common to methotrexate (MTX) to allow ready comparison both with earlier inhibitor classes and between those containing different oxidation states of phosphorus. It is known, however, that changing the heterocycle within one class of FPGS inhibitor can lead to greater potency and even increased specificity [19]. Therefore, we have prepared individual diastereomers of the phosphinate-containing dipeptide mimics and each diastereomer has been coupled to three different heterocycles, including 4-amino-4-deoxy-10-methylpteroate (**4**) [20], pterate (**5**), and 5,10-dideazatetrahydropteroate (**6**) (Figure 2). Inhibitory potency of each diastereomer and a structure-activity relationship for the heterocycles has been determined and the results are reported herein.

In addition to the phosphinates designed to mimic the tetrahedral intermediate formed during FPGS-catalyzed ligation of the first glutamate, phosphinate mimics of intermediates formed during subsequent ligations were also of interest. Thus, the synthesis of **7** (Figure 2), a pteroyl derivative that incorporates elements to mimic the intermediate formed during ligation of the second glutamate is described (Supplementary Material). The inhibitory properties of this analog are also reported.

Materials and methods

Materials

Common chemicals were reagent grade or higher. MTX was a generous gift of Immunex (Amgen; Seattle, WA). Aminopterin (AMT) was from Sigma Chemical Co. (St. Louis, Mo).

¹Abbreviations used: ACN, acetonitrile; AMPte, 4-amino-4-deoxy-10-methylpteroate; AMT, aminopterin (4-amino-4-deoxy-pteroylglutamic acid); CPG2, carboxypeptidase G2 (EC 3.4.17.11); DDAH₄Pte, (6R)-5,10-dideazatetrahydropteroate; DDAH₄PteGlu or DDATHF, (6R)-5,10-dideazatetrahydropteroylglutamate; FPGS, folylpolyglutamate synthetase (EC 6.3.2.17); Glu, glutamic acid; GCP II, glutamate carboxypeptidase II (EC 3.4.17.21); MTX, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid); Pte, pterate.

The phosphorus-containing diastereomeric pseudo-dipeptide and -tripeptide (phosphinate) precursors were prepared as described by Bartley and Coward [20]. The required pteroyl azide [11], 4-amino-4-deoxy-10-methylpteroyl (MTX) azide [20], and 5,10-dideazatetrahydropteroyl (DDATHF) azide [21] were prepared as described. Coupling of the phosphinate precursor to the appropriate azide and subsequent work up was carried out similarly to that described for the MTX azide [20]. Details of the synthetic procedure and characterization of intermediates and products (**5–7**) are presented in Supplementary Material associated with this article available in the online version, at doi: #####. Suitably blocked –L-Glu- γ -L-Glu and –L-Glu- γ -D-Glu dipeptides were synthesized as described previously for oligo- γ -glutamates consisting of L-Glu [21] and coupled to the various azides (above) to afford **8–10** as described previously [21] and in the Supplementary Material. Analog solutions for all experiments were standardized using extinction coefficients from the literature. It was assumed that the phosphinates had extinction coefficients identical to the heterocycle containing Glu. Since the spectra of the phosphinates were identical or nearly identical to those of the Glu-containing species, this assumption is warranted.

Enzymes and assays

Recombinant human cytosolic FPGS was expressed in Sf9 cells and purified as described previously [12]. FPGS substrate activity of analogs was determined as described [12]. Time linearity was verified at low and high substrate concentrations in all assays. Because of the low K_m for DDATHF, the concentration of [^3H]Glu in assays was decreased to 2 mM (from 4 mM) and the DE-52 column wash solution was adjusted to 130 mM NaCl (from 110 mM NaCl); this concentration of NaCl was verified to allow quantitative retention of DDATHF and thus its polyglutamates would also be retained. Inhibitory potency (IC_{50}) of analogs against rhcFPGS was determined as described [10]. Inhibition constants K_{ii} and K_{is} [22] for rhcFPGS were determined [10] using MTX as the variable substrate (30–1000 μM) and two levels of inhibitor yielding $\approx 25\%$ and $\approx 50\%$ inhibition, respectively; these were compared to an identical control lacking inhibitor. In some experiments, DDATHF was used as the variable substrate (1–25 μM) to verify that the class of variable substrate did not affect the potency and inhibitory mechanism. Pure carboxypeptidase G2 (CPG2) from *Pseudomonas* sp. strain RS-216 was a gift of the late Dr. Roger Sherwood (Microbial Technology Laboratory, PHLS Centre for Applied Microbiology and Research, Porton Down, England) [23]. Cloned soluble recombinant glutamate carboxypeptidase II (GCP II), obtained from Dr. Takashi Tsukamoto (Guilford Pharmaceuticals) was assayed as described [24].

HPLC methods

Semi-preparative HPLC purification of the phosphinate pseudopeptides, **4–7** and the L-Glu- γ -D-Glu derivatives, **8–10**, employed an HPLC system (Rainin, Woburn MA) that consisted of two Rabbit Model HPX pumps fitted with 50 mL/min pump heads, a dynamic mixer, and a single wavelength UV detector. All separations utilized a Varian Dynamax 21.4 $\times$ 250 mm, Microsorb 60–8, C18 column with a flow rate of 21 mL/min. Purification conditions were as follows: Eluent A – 20 mM ammonium acetate, pH = 6.5, in ddH₂O; Eluent B – MeOH; Gradient – 0 min, 5% B; 10 min, 5% B; 20 min, 50% B; 25 min 50%B. Details on the purification of each compound can be found in the Supplementary Material (doi: #####).

Further semi-preparative HPLC purification of Pte-L-Glu- γ -D-Glu was performed on a 4.6 $\times$ 250 mm analytical C₁₈ column (5 μ , Rainin Microsorb, Varian, Walnut Creek, CA) using 0.1 M Na-acetate, pH 5.5 containing 4% acetonitrile (ACN) isocratically at 1 mL/min for 30 min and injecting no more than 2.4 $\mu\text{mol}/400 \mu\text{L}$ injection using a 500 μL loop. Individual 1 min (1 mL) fractions were collected and each fraction was subjected to analytical HPLC as described above (15 min run time) to determine the level of putative Pte-L-Glu- γ -L-Glu contamination.

Highly purified fractions of Pte-L-Glu- γ -D-Glu from multiple runs were pooled, exchanged into ammonium bicarbonate buffer by DEAE-cellulose (DE-23; Whatman, Inc., Florham Park, NJ) chromatography, lyophilized to remove salts, and re-analyzed. The original synthetic Pte-L-Glu- γ -D-Glu contained $\approx 0.7\%$ of putative Pte-L-Glu- γ -L-Glu, while the final purified material contained $<0.01\%$. Remaining fractions enriched for the putative Pte-L-Glu- γ -L-Glu contaminant were pooled separately, processed as above, and also analyzed by HPLC; the final material contained $\approx 2.1\%$ of the contaminant. Analysis of GCP II hydrolysis products used a modified system employing 0.1 M Na-acetate, pH 5.5 containing 3% ACN isocratically for 15 min and then a 15 min linear gradient to 13% ACN to separate the two diastereomers while still allowing detection of the PteGlu hydrolysis product; typical R_t were L,L (16.9 min), L,D (19.4 min), and PteGlu (27.7 min).

Results and discussion

Inhibition of rhcFPGS by phosphinate-containing dipeptide mimics

Four separate diastereomeric pairs (low R_f and high R_f) of the phosphinic acid-containing pseudopeptide were prepared, each pair of which contained a different heterocycle and/or chain length (Table 1). The pairs based on MTX (AMPte, **4**) were of immediate interest because this heterocycle has been used as a standard in earlier FPGS inhibitor design efforts. We previously showed that the IC_{50} for the mixture containing all four diastereomers was 8 ± 1 nM [12]; in the current studies, the value obtained was 13.6 ± 1.3 nM, indicating good agreement. Comparing the two diastereomers containing this heterocycle, the high R_f species was about 13-fold more potent than the low R_f species (IC_{50} , Table 1). Current knowledge of FPGS stereospecificity thus suggests that the high R_f species is analogous to the natural L-Glu- γ -L-Glu configuration. Further characterization revealed that the high R_f species was a competitive inhibitor of rhcFPGS ($K_{is} \ll K_{ii}$) with a low nM K_{is} . A replot of K_m/V_{max} vs. [inhibitor] was linear and thus inhibition was linear competitive [22]. We did not observe time-dependence in inhibitory potency of any phosphinate isomer when we compared residual activity at $t_{0.5}$ and t , similar to our previous studies with mixtures of diastereomers [12]. However, because of the lengthy (90–120 min) reaction time course required to observe FPGS activity, we cannot preclude time-dependent events that occur in the first few minutes of the reaction. It is well known that FPGS exhibits significant ATPase activity [25–27]. For this reason, it was not possible to demonstrate ATP-dependent, FPGS-catalyzed formation of a phosphorylated phosphinate, **3** (Figure 1) [12].

Substitution of other heterocycles (pteroate (Pte) or 5,10-dideazatetrahydropteroate (DDAH₄Pte)) altered the absolute potency, but did not change the relationship between potency of low and high R_f species (IC_{50} , Table 1). In each case, the high R_f species was significantly more potent than the low R_f species. The most potent inhibitor was the high R_f species containing the DDAH₄Pte heterocycle. The high R_f diastereomer was kinetically characterized as an inhibitor with both MTX and DDAH₄PteGlu (DDATHF) as the variable substrate. Similar inhibitor constants were obtained as expected if a single kinetic site is involved (Table 1) and the inhibition was linear competitive regardless of the substrate used. The high potency of the DDAH₄Pte-containing pseudopeptide mimic of the tetrahedral intermediate formed during the biosynthesis of the Glu- γ -Glu product was anticipated based on the low K_m value (0.65 μ M; [21]) of the corresponding Glu- γ -Glu dipeptide as an FPGS substrate. However, the relative potencies of the other high R_f dipeptide species did not parallel the K_m values of their corresponding Glu- γ -Glu dipeptides as FPGS substrates (PteGlu₂, $K_m = 16$ μ M [28] and AMPteGlu₂, $K_m = 63$ μ M [10]). The following values of K_m/K_{is} were determined for each L-Glu- γ -L-Glu dipeptide substrate and its corresponding pseudopeptide inhibitor: AMPte, 27.4×10^3 ; Pte, 3.4×10^3 , and DDAH₄Pte, 0.7×10^3 . This is in contrast to an earlier series of substantially weaker FPGS inhibitors in which the intrinsic Glu was replaced by ornithine

[19], where their relative potencies mirrored the efficiencies of the Glu-containing parents as FPGS substrates. In the current research, values of K_i reflect events, probably including protein dynamics, associated with binding of an analog of an unstable reaction intermediate. In contrast, values of K_m reflect ground-state events during the binding and turnover of substrates. Presumably, the ornithine derivatives also bind as ground-state analogs. In related research on glutathionyl spermidine synthetase/amidase (EC 6.3.1.8/3.5.1.78), a bifunctional protein that encodes both an ATP-dependent ligase and a cysteine amidase, binding of a similar phosphinate-containing “tetrahedral mimic” to the synthetase domain has a marked effect on both the catalytic activity [29] and structure [18] of the amidase domain. This suggests that the primary source of binding energy for these analogs comes from the phosphinate-containing pseudodipeptide, and that these mimics of unstable intermediates formed during catalysis are able to recruit distal domains to the ligand-protein interactions.

The lower potency of the Pte-(γ -Glu)₃ mimic **7** with the phosphinate in the terminal position relative to the Pte-(γ -Glu)₂ mimic was unanticipated in the initial design since PteGlu₂ (K_m = 16 μ M [28]) has a K_m value similar to that of PteGlu₃ (K_m = 20 μ M [28]) which suggests the possibility that their affinities (K_d) could also be similar. However, subsequent to the synthesis of **7**, detailed kinetic studies with DDATHF provided evidence for the processive ligation of multiple glutamates at a low but saturating ($\sim 10 K_m$) folate substrate concentration [30]. Thus, the first γ -ligation is fast followed by slower steady-state elongation to higher chain length products. It would be expected that the longer chain oligoglutamates would not have access to the FPGS active site during processive ligation of multiple glutamates. This restrictive access of higher chain length folate substrates or analogs to the FPGS active site is consistent with the markedly decreased inhibitory activity of the Glu- γ -Glu- γ -Glu analog, **7**. However, only one member of this class was prepared, and further work will be required to determine if this is a general feature of pseudotripeptide mimics, is specific for the Pte heterocycle, or is a function of the position of the phosphinate tetrahedral mimic.

These studies demonstrate that very potent mechanism-based inhibitors of purified human FPGS employing a variety of heterocycles can be designed and synthesized. Because phosphinate-containing pseudodipeptide inhibitors such as **4–8** apparently do not enter human cells [12], the next task is to transform these inhibitors into cell-permeable drugs. In this regard, at least two approaches can be envisioned. The first is to synthesize prodrugs that will allow these hydrophilic moieties to enter cells for subsequent activation. An initial attempt in this regard utilized readily hydrolyzable pivaloyloxymethyl (POM) esters that have been used previously to deliver several hydrophilic drugs [31]. Unfortunately, previously unrecognized relatively rapid ($t_{0.5}$ = 30 min) hydrolysis of the esters in medium or solvent was documented. However, the product of this rapid intramolecular reaction is not the parent drug [31]. Further effort will be required to generate a suitable prodrug. The second approach to developing cell-permeable FPGS inhibitors involves determining the minimum structure of the phosphinate-containing pseudodipeptide that is required for potent inhibition. This approach may lead to new inhibitors that possess an intrinsically higher ability to enter cells either by diffusion or by mediated processes. Investigations of both approaches are continuing.

rhcFPGS substrate activity of L-Glu- γ -L-Glu and L-Glu- γ -D-Glu-containing pteroyl substrates

Current evidence suggests that FPGS is highly stereospecific for L-Glu (or close L-Glu structural analogs); this evidence is direct for the position occupied by the intrinsic Glu (position 1; [25,32,33]), but indirect for the first γ -Glu (position 2; [34]). Thus, the data of Table 1 were unexpected because the low R_f species, which presumably mimic the unnatural L-Glu- γ -D-Glu configurations, remain highly potent FPGS inhibitors. The high affinity of this unnatural configuration suggested that the substrate activity of pteroyl-L-Glu- γ -D-Glu species should be directly evaluated. Accordingly, the L-Glu- γ -L-Glu and L-Glu- γ -D-Glu dipeptides

conjugated to the Pte, AMPte, and DDAH₄Pte heterocycles were synthesized (Supplementary Material; doi: #####) for characterization as FPGS substrates. Because of limitations in the supply of purified FPGS, only single substrate concentrations were evaluated, which were equivalent to 4 – 10 K_m for the respective L-Glu-γ-L-Glu conjugates of each heterocycle [10,21,28]. The L-Glu-γ-L-Glu conjugates were confirmed as active FPGS substrate (Table 2). In contrast, the L-Glu-γ-D-Glu conjugates of Pte and AMPte showed no significant activity (Table 2). However, the K_m values of the corresponding L-Glu-γ-L-Glu conjugates are relatively high (vide supra), and with the limited amount of compound and FPGS available, we cannot preclude low activity for the L-Glu-γ-D-Glu conjugates that might be apparent only at very high substrate concentrations. Consistent with this hypothesis, DDAH₄Pte-L-Glu-γ-D-Glu showed activity equivalent to DDAH₄Pte-L-Glu-γ-L-Glu when both were present at 100 μM (200 K_m) (Table 2). A full concentration-dependence curve employing similarly pure human FPGS (Tsai and Coward, unpublished results) showed similar results and yielded a K_m value for DDAH₄Pte-L-Glu-γ-D-Glu of ≈9 μM, which was 19-fold higher than that of DDAH₄Pte-L-Glu-γ-L-Glu (K_m, 0.5 μM) in the same experiment; in addition, the V_{max} of DDAH₄Pte-L-Glu-γ-D-Glu was ≈66% that of DDAH₄Pte-L-Glu-γ-L-Glu, thus leading to V_{max}/K_m values of $3.5 \times 10^{-3} \text{s}^{-1}$ and $1.1 \times 10^{-4} \text{s}^{-1}$ for the L-Glu-γ-L-Glu and L-Glu-γ-D-Glu isopeptides, respectively.

Because the K_m of the DDAH₄Pte-containing L,L species is very low relative to the L,D species, the possibility that DDAH₄Pte-L-Glu-γ-D-Glu contained low levels of its L,L diastereomer was considered. Consistent with this suggestion was the finding (Table 2) that the product distribution at a high concentration of DDAH₄Pte-L-Glu-γ-D-Glu was similar to that observed at the low concentration of DDAH₄Pte-L-Glu-γ-L-Glu and contained multiple products, including those having long chain length. We previously noted [21,25,30] the inverse concentration-dependence of product length for FPGS such that low concentrations of effective substrates tend to promote synthesis of longer products, while higher concentrations tend to produce only one product containing a single additional γ-Glu. Thus a nonsubstrate containing a low concentration contaminant that is an efficient FPGS substrate would tend to produce multiple products including those containing long chain lengths. The reversed-phase HPLC system employed to determine general purity did not resolve DDAH₄Pte-L-Glu-γ-D-Glu from DDAH₄Pte-L-Glu-γ-L-Glu (or AMPte-L-Glu-γ-D-Glu from AMPA-L-Glu-γ-L-Glu) despite extensive efforts to develop modified methods to effect these separations. However, Pte-L-Glu-γ-L-Glu (R_t 18.8 min) and Pte-L-Glu-γ-D-Glu (R_t 22.4 min) were nearly baseline resolved. Because the same dipeptide precursors are conjugated to each heterocycle, if the presence of an L,L diastereomer contaminant can be demonstrated in any L,D diastereomer, it suggests that it is present in all. Accordingly, we focused on determining stereochemical purity of Pte-L-Glu-γ-D-Glu.

Initial HPLC analysis showed that a small (0.7% by area) peak eluting at the R_t of Pte-L-Glu-γ-L-Glu was present in synthetic Pte-L-Glu-γ-D-Glu. Co-injection of Pte-L-Glu-γ-D-Glu with a trace of Pte-L-Glu-γ-L-Glu demonstrated the co-elution of the contaminant peak with Pte-L-Glu-γ-L-Glu. To corroborate this finding, we explored sensitivity of these peaks to enzymatic hydrolysis. Initially we attempted to use carboxypeptidase G2 (CPG2) to hydrolyze L,L-containing dipeptides because the closely related enzyme CPG1 was reported to catalyze hydrolysis of pteroyl-(γ-L-Glu)₃ [35]. However, under conditions where a pteroyl-L-Glu itself was quantitatively hydrolyzed in 30 min (half-time, ≈6 min), CPG2 failed to hydrolyze the corresponding pteroyl-L-Glu-γ-L-Glu (<0.8% hydrolysis in 60 min at 10× CPG2). Consequently, we employed a cloned, purified soluble glutamate carboxypeptidase II (GCP II; also known as PSMA or NAALadase; [36]), which possesses γ-L-glutamyl hydrolase activity [36,37], but which does not hydrolyze AMPte-L-Glu-γ-D-Glu [37]. To increase sensitivity, a side-fraction obtained during HPLC purification of Pte-L-Glu-γ-D-Glu (Methods) containing ≈2% of the putative L,L contaminant was employed. HPLC analysis

after GCP II hydrolysis of this material showed that the main Pte-L-Glu- γ -D-Glu peak was not hydrolyzed; the putative Pte-L-Glu- γ -L-Glu contaminant, however, was reduced >96% while producing a concomitant increase in a peak with the retention time of the expected hydrolysis product PteGlu. Similar results were obtained following PSMA treatment of HPLC-purified Pte-L-Glu- γ -D-Glu that contained <0.01% of the putative L,L material. These data are consistent with the contaminant being the L,L-containing diastereomer. Since the same dipeptide was used to synthesize DDAH₄Pte-L-Glu- γ -D-Glu, the data further suggest that this diastereomer is contaminated with as much as 0.7% DDAH₄Pte-L-Glu- γ -L-Glu and this level could produce the FPGS substrate activity observed (Table 2) because of the low K_m of the L,L diastereomer. Our results show that caution must be used when interpreting the results of studies of the stereospecificity of FPGS, especially when one isomer has an especially low K_m value. The occurrence of contaminating diastereomers of antifolate γ -dipeptides has been noted previously [34], but the biochemical consequences of these contaminants, especially with respect to FPGS activity, were not reported. Returning to the phosphinate-containing pseudopeptides, the two stereocenters of the final products were derived from, respectively, commercial L-glutamic acid to yield the 2S (L-) configuration at the N-terminal glutamate and a stereorandom synthesis of the C-terminal moiety. Purification of the diastereomeric mixture provided the “high R_f ” and “low R_f ” products that have been evaluated as reported in this paper. Based on the route of synthesis, contamination of pseudopeptides **4–6** with other stereoisomers is highly unlikely.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to acknowledge Mr. Sanyo Tsai for assisting in the synthesis of the blocked L-Glu- γ -L-Glu and L-Glu- γ -D-Glu dipeptides and their conjugation to the various heterocycles and also for the initial kinetic analysis of the DDAH₄Pte diastereomers as FPGS substrates. The authors thank Dr. Takashi Tsukamoto of Guilford Pharmaceuticals for his generous gift of purified recombinant human GCP II. This work was supported by grants CA43500 (JJM), Cancer Center Support Grant CA16056 to Roswell Park Cancer Institute, and CA28097 (JKC). D.M.B. was a trainee in the Pharmaceutical Sciences Training Program supported by grant GM007767 from National Institutes of General Medical Sciences (NIGMS). J.W.T. was a trainee of the Michigan Chemistry-Biology Interface Training Program, supported in part by grant GM008597 from NIGMS. J.W.T. was the recipient of a Fred W. Lyons Fellowship from the College of Pharmacy, University of Michigan, and a fellowship from the American Foundation for Pharmaceutical Education. The contents are solely the responsibility of the authors and do not necessarily represent the official views of NCI or NIGMS.

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Figure 1. Proposed tetrahedral intermediate for FPGS-catalyzed ligation (**1**), phosphorus-containing pseudo-peptide “tetrahedral mimics” (**2**), and a possible phosphorylated product of the phosphinate pseudo-peptide (**3**).

Figure 2.

Phosphinate pseudopeptide mimic of tetrahedral intermediate, **1**, for initial (**4–6**) and for subsequent (**7**) glutamate ligation.

Table 1

Inhibition of recombinant human cytosolic FPGS by phosphinic acid-containing pseudopeptides. For reference, the original MTX-containing species containing all diastereomers (4 isomers) was included in each determination and yielded an IC_{50} of 13.6 ± 1.3 nM ($n = 9$). The K_m for MTX as the substrate in K_i determination studies was 65 ± 7 μ M ($n = 10$). Values presented are average $\pm$ range for duplicate experiments and average $\pm$ S.D. for $n \geq 3$.

Heterocycle (R) & Structure Number		n	Low R_f		High R_f	
			IC_{50} , nM	K_{is} , nM	IC_{50} , nM	K_{ip} , nM
0	45 ± 0.3 ($n = 2$)	2.3 ± 0.4 ($n = 3$)	3.5 ± 0.3 ($n = 3$)	36 ± 4 ($n = 3$)
4				

Heterocycle (R) & Structure Number		Low R _f		High R _f	
n =		IC ₅₀ , nM	K _i , nM	IC ₅₀ , nM	K _i , nM
0		65 ± 8 (n = 2)	4.7 ± 1.6 (n = 3)	6.5 ± 0.4 (n = 2)	38 ± 6 (n = 3)
1		576 ± 26 (n = 2)	34 ± 5 (n = 2)	98 ± 14 (n = 2)	1080 ± 220 (n = 2)

Heterocycle (R) & Structure Number				
n =	Low R_f	High R_f		
	IC ₅₀ , nM	K _{is} , nM	K _{ip} , nM
51 ± 5 (n = 2)	1.7 ± 0.2 (n = 2) (3.0 ± 1.0) ^a (n = 2)	0.9 ± 0.3 (n = 2) (1.0 ± 0.1) ^b (n = 2)	
0			11 ± 4 (n = 2) (136 ± 90) ^b (n = 2)	

^aDDATHF (2 μM) was used as the fixed substrate.

^bVariable substrate, (6R)-DDATHF (K_m, 1.8 ± 0.2 μM; this research); MTX is the variable substrate for all other K_i determinations.

FPGS substrate activity of folates or antifolates containing -L-Glu- γ -L-Glu or -L-Glu- γ -D-Glu dipeptides. FPGS activity was assayed at the indicated concentration of each compound in two separate experiments (each point in duplicate) as described in Methods; results were similar in both experiments.

Table 2

Heterocycle	Heterocycle Abbreviation	Concentration, μ	-L-Glu- γ -L-Glu pmol [3 H]Glu incorp	-L-Glu- γ -D-Glu pmol [3 H]Glu incorp
AMPte	250 (4 K_m)	188 $\pm$ 9 ^a	2.5 $\pm$ 2.5 ^a
Pte	160 (10 K_m)	690 $\pm$ 2 ^b	0 $\pm$ 0 ^b
DDAH ₄ Pte	5 (10 K_m) 100 (200 K_m)	185 $\pm$ 3 ^c 192 $\pm$ 14 ^c	ND ^d 184 $\pm$ 1 ^c

^a 500 μ M AMPteGlu (MTX) was included as a control and led to the incorporation of 1050 $\pm$ 40 pmol [3 H]Glu incorporated in the experiments shown. With AMPte-L-Glu- γ -L-Glu as substrate, only AMPte-L-Glu- γ -L-Glu₂ was identified by HPLC product analysis.

^b 600 μ M PteGlu (folic acid) was included as a control and led to the incorporation of 660 $\pm$ 20 pmol [3 H]Glu incorporated in the experiments shown. With Pte-L-Glu- γ -L-Glu as substrate, Pte-L-Glu- γ -L-Glu₂ was the major product identified by HPLC analysis with only a trace of Pte-L-Glu- γ -L-Glu₃.

^c 20 μ M DDAH₄PteGlu (DDATHF) was used as a control and led to the incorporation of 185 $\pm$ 3 pmol [3 H]Glu incorporated in the experiments shown. With 5 μ M DDAH₄Pte-L-Glu- γ -L-Glu, the products were DDAH₄Pte-L-Glu- γ -L-Glu₂ (94%) and DDAH₄Pte-L-Glu- γ -L-Glu₃ (6%). At 100 μ M DDAH₄Pte-L-Glu- γ -L-Glu, only DDAH₄Pte-L-Glu- γ -L-Glu₂ was present. At 100 μ M DDAH₄Pte-L-Glu- γ -D-Glu the products were DDAH₄Pte-L-Glu- γ -L-Glu₂ (79%), DDAH₄Pte-L-Glu- γ -L-Glu₃ (15%), and DDAH₄Pte-L-Glu- γ -L-Glu₄ (5%).

^d ND = Not determined