A Mechanism for the Addition of Multiple Moles of Glutamate by Folylpolyglutamate Synthetase

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The role of the α-carboxyl group in methotrexate (MeAPA-Glu) and the γ-glutamate derivative of methotrexate (MeAPA-Glu-Glu) in the reaction catalyzed by folylpolyglutamate synthetase (FPGS) has been investigated. MeAPA-Glu and MeAPA-Glu-Glu were accepted as substrates by the same FPGS species contained in an $(NH_4)_2SO_4$ precipitate of mouse liver protein, as judged by a lack of additivity of product formation at saturating concentrations of both substrates. MeAPA-Gaba, the MeAPA-Glu analogue lacking an α-carboxyl, was inactive as a substrate for this enzyme as was MeAPA-Glu-Gaba, the analogue of MeAPA-Glu-Glu that lacked the α-carboxyl of the terminal glutamic acid. However, MeAPA-Gaba-Glu, the analogue of MeAPA-Glu-Glu without an α-carboxyl on the first glutamic acid, had activity as a substrate for FPGS that approached that of MeAPA-Glu-Glu. These results suggest that the α-carboxyl is essential for the binding of folyl monoglutamates to FPGS in the correct orientation to allow catalysis. Moreover, the binding of the terminal α-carboxyl of folyl oligoglutamates to the same residue(s) responsible for the binding of the α-carboxyl of folyl monoglutamates would allow correct positioning of the terminal γ-carboxyl of the chain for reaction. This binding mechanism would be compatible with the utilization of a single enzyme species for the addition of glutamate to the monoglutamate or oligoglutamate forms of folates and folate analogues.

The folate content of mammalian tissues has been shown by a variety of techniques to be principally in the form of γ-linked glutamyl conjugates containing several moles of glutamic acid per mole of pteridine.[2-6] In rodents and man, there is an average of 5–6 mol of glutamate per mole of folate.[4-6] The enzyme(s) that synthesizes this class of compounds from the folyl monoglutamates, i.e., folylpolyglutamate synthetase(s) (FPGS),[1] has/have been purified to homogeneity from bacteria[7] and to a much lesser extent from rodent[8,9] and hog[10] liver and Chinese hamster ovary cells.[11] The kinetics of the reaction catalyzed by the bacterial enzyme are incompatible with any mechanism involving the addition of several moles of glutamate to the folate nucleus without intermediate release of substrate from the active site.[12] Likewise, the product of the mammalian FPGS reaction with monoglutamyl substrate has been chromatographically characterized as a pteroyl diglutamate when the substrate was present at saturating conditions and as a mixture of oligoglutamates at low substrate concentrations.[8,9,11] This suggests that mammalian FPGS operates by a mechanism that involves release of substrate from the active site after each catalytic event. However, mammalian FPGS has not yet been extensively purified due, in part, to a limiting enzyme instability.[8,9] Hence, it has not been possible to unequivocally establish whether the addition of glutamic acid to pteroyl mono-, di-, tri-, and tetraglutamates is catalyzed by a single or by multiple enzyme species in animal tissues. It should be noted, however, that genetic analysis of a mutant line of Chinese hamster ovary cells lacking FPGS indicates that the loss of the ability to add glutamate to pteroyl monoglutamates was due to mutation at a single genetic locus.[11,14]

We have recently compared the substrate activity of a preparation of mouse liver FPGS for a series of folate analogues differing by a single structural change in various positions of the molecule.[9,15] During that study, we found that esterification of the α-carboxyl in the folate analogue methotrexate (MeAPA-Glu) resulted in substantial loss of substrate activity for FPGS. We now present evidence that it is the α-carboxyl of the *terminal* glutamic acid of an oligoglutamate, rather than the α-carboxyl of the first glutamate per se that is required for substrate activity. This suggests a mechanism of binding of pteroyl mono- and oligoglutamates to FPGS that could explain the utilization of substrates of such divergent structure by a single enzyme species.

Results and Discussion

The structures of the derivatives of MeAPA-Glu and MeAPA-Glu-Glu used in these experiments, MeAPA-Gaba (**2**), MeAPA-Gaba-Glu (**3**), and MeAPA-Glu-Gaba (**4**), and their synthesis from 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA, **5**) are shown in Chart I. Activation of the carboxyl group in **5** was achieved with diethyl phosphorocyanidate as previously reported,[16] and the mixed carboxylic-phosphoric anhydride intermediate was allowed to react in situ with the *O,N*-bis(trimethylsilyl) derivative of 4-aminobutyric acid to give, upon aqueous workup, a

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(1) The abbreviations used were as follows: folylpolyglutamate synthetase, FPGS; methotrexate, MeAPA-Glu; folic acid, PteGlu; pteroyl triglutamate, $PteGlu_3$; the structures of various conjugates of 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA), γ-aminobutyric acid (Gaba), and glutamic acid (Glu) are shown in Chart I.

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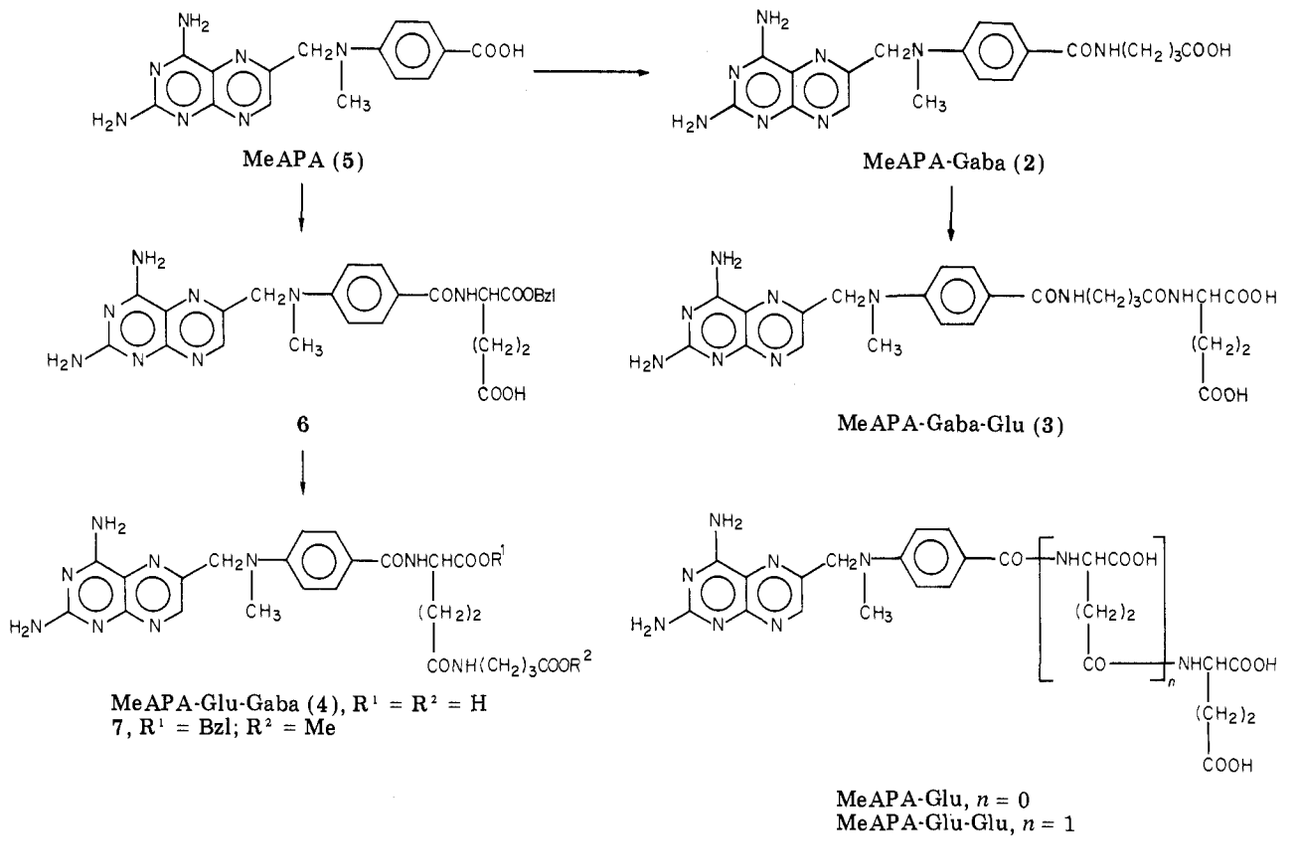
Chart I



92% yield of analytically pure **2**. Earlier workers[17] reported only a 5–10% yield of **2** by a route involving condensation of ethyl 4-[*N*-[*p*-(methylamino)benzoyl]amino]butyrate and 2,4-diamino-6-(chloromethyl)pteridine, followed by saponification and purification of the crude acid via its magnesium salt. The present method is clearly superior in yield and convenience. Attachment of other amino acids to the terminal carboxyl group in **2** is readily achieved by conventional methods of peptide synthesis, as illustrated in this instance by the formation of **3** in 54% yield from the *N,O,O*-tris(trimethylsilyl) derivative of L-glutamic acid. Since **3**, like MeAPA-Glu, contains two carboxyl groups, it can be separated easily from either **5** or **2** by chromatography.

Also shown in Chart I is methotrexate α-benzyl ester (**6**), which was obtained in 94% yield from **5**, diethyl phosphorocyanidate, and α-benzyl-L-glutamic acid by the method that has been recently described.[18,19] Activation of the γ-carboxyl group in **6** with diphenylphosphoryl azide, followed by addition of methyl 4-aminobutyrate and saponification of the resultant diester **7** with barium hydroxide in aqueous ethanol (1:1), afforded **4** in approximately 50% overall yield after ion-exchange chromatography on DEAE-cellulose, which readily separates **4** from either **5** or **6**.

In these experiments, a minimally purified (ca. 12-fold with 70% recovery) enzyme preparation was used to avoid interfering side reactions[9] or loss of a FPGS species during purification. Conjugase (i.e., folyloligoglutamate hydrolase) activity could not be detected in these preparations under the conditions used for assay of FPGS activity (pH 8.5 buffer without Zn^{2+}). Under conditions optimal for conjugase assay (pH 4.5 buffer with 1 mM Zn^{2+}), these preparations had conjugase activities <2% that of the activity of FPGS. The reaction catalyzed by this $(NH_4)_2SO_4$-precipitated fraction was previously shown to be linear for at least 1 h and the products isolated by our procedure have been characterized as folyl oligoglutamates by chromatography on DEAE-cellulose and Sephadex G-25 and by reversed-phase HPLC.[9,13] As shown in Figure 1, this preparation of mouse liver FPGS catalyzed the addition of glutamic acid to both MeAPA-Glu and MeAPA-Glu-Glu. However, the maximal rate of enzyme reaction and the concentration of substrate required to reach half-maximal rates of reaction differed substantially between these compounds. In the experiments reported here, MeAPA-Glu-Glu at saturating concentrations was converted to product at 41 ± 5% of the rate observed with 500 μM MeAPA-Glu. Half-maximal reaction was observed in the presence of 163 ± 31 μM MeAPA-Glu (eight determinations) and in the presence of 47 ± 0.9 μM MeAPA-Glu-Glu (two determinations). Parallel differences in these parameters have been observed in the comparison of PteGlu and $PteGlu_3$ with this preparation of FPGS.[9] Compound **2**, which differs from MeAPA-Glu only by deletion of the α-carboxyl, was not a substrate for FPGS at any concentration up to 1 mM (Figure 1A); the amount of product formed at 0.5–1.0 mM **2** was 0.6 ± 0.6% (three determinations) of that formed from a control incubation using MeAPA-Glu at 500 μM as substrate. Hence, it is clear that the α-carboxyl of MeAPA-Glu is essential for FPGS activity. Likewise, formation of charcoal-adsorbable products from **4** under conditions of the FPGS assay were negligible (<3.0%) relative to the reaction rate observed with a control of MeAPA-Glu in the same experiment (Figure 1B). We have not excluded the possibility that the low reaction rate seen with **4** may represent contamination of substrate with trace amounts of MeAPA-Glu.

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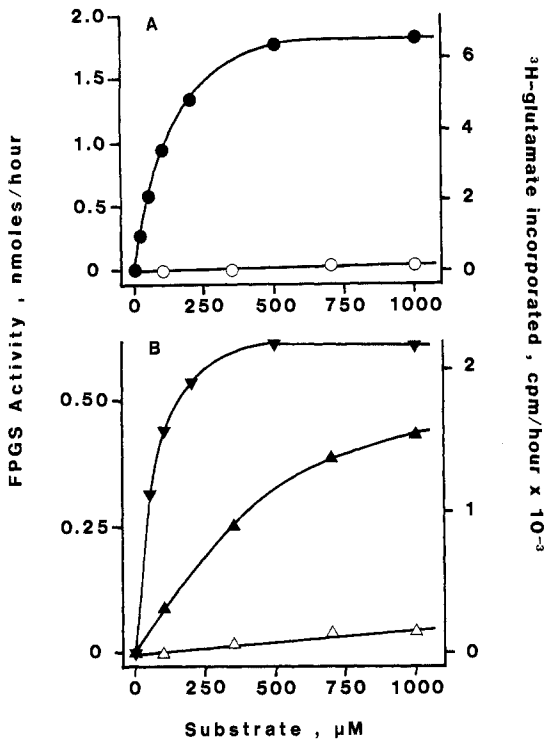

Figure 1. Substrate activity of analogues of MeAPA-Glu and MeAPA-Glu-Glu for mouse liver FPGS. $(NH_4)_2SO_4$-precipitated mouse cytosol protein was desalted and incubated with the indicated concentrations of MeAPA-Glu (●), **2** (○), MeAPA-Glu-Glu (▼), **3** (▲), or **4** (△) for 1 h at 37 °C as described in the Experimental Section. Each symbol represents the mean of two determinations from a representative experiment.

Table I. Lack of Additivity of FPGS Reaction Rates with MeAPA-Glu and MeAPA-Glu-Glu[a]

substrate	[³H]-L-glutamate incorporated, nmol/h
MeAPA-Glu	1.71 ± 0.06
MeAPA-Glu-Glu	0.70 ± 0.01
MeAPA-Glu + MeAPA-Glu-Glu	1.23 ± 0.02

[a] The designated compounds were incubated with $(NH_4)_2SO_4$-precipitated mouse liver FPGS for 1 h at a concentration of 500 μM. The reaction rates reported are means ± SD of triplicate samples from a representative experiment.

Compound **3**, in contrast to **2** and **4**, has significant activity as a substrate for mouse liver FPGS (Figure 1B). Computer analysis[20] of the best rectangular hyperbola fit to these data indicates that the maximal velocities that would be obtained with **3** and MeAPA-Glu-Glu at saturation were not significantly different. However, the concentrations of **3** that half-saturated this reaction was substantially higher than that of MeAPA-Glu-Glu (Figure 1B).

If the addition of glutamic acid to MeAPA-Glu was catalyzed by the same FPGS species as that which reacted with MeAPA-Glu-Glu, it would be expected that the simultaneous presence of saturating concentrations of both substrates would result in the formation of charcoal-adsorbable products at a rate intermediate between the V_{max} values observed with either substrate alone. If different enzyme activities were acting on these substrates, on the other hand, additivity of reaction rates would be expected. The data of Table I demonstrate that the rate of product formation was mutually exclusive and, hence, suggests that only one enzyme was involved in both reactions.

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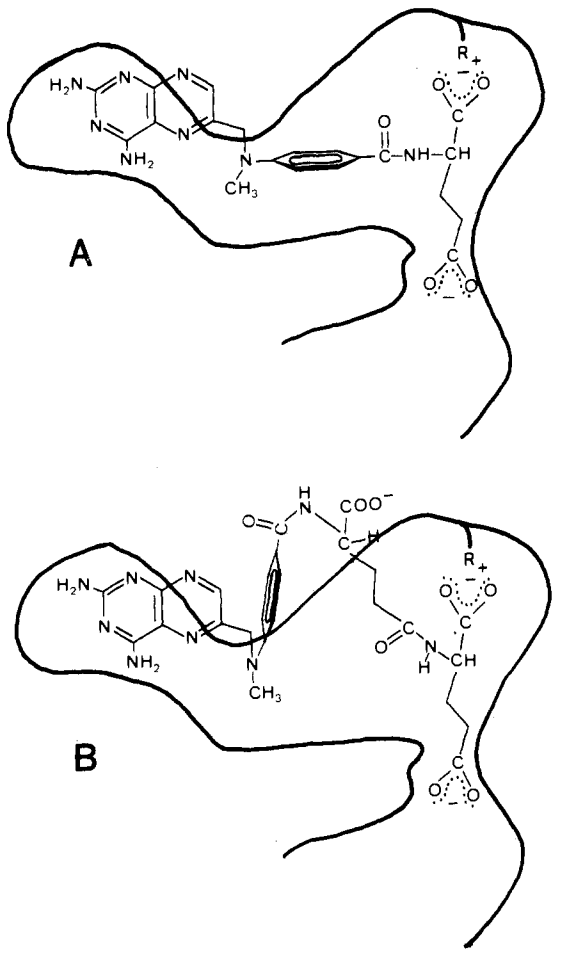

Figure 2. Proposed geometry of binding of MeAPA-Glu (A) and MeAPA-Glu-Glu (B) in the active site of FPGS. The heavy line indicates the enzyme surface. Binding of substrate in the active site involves the pteridine ring and interaction of the α-carboxyl of the terminal glutamic acid with a cationic residue (R^+). Under these conditions, the γ-carboxyl lies close to other residue(s) involved in catalysis.

We have previously shown that the number of methylene groups between the asymmetric α-carbon and the terminal carboxyl in MeAPA-Glu analogues cannot be altered without major impact on the substrate activity with mouse liver FPGS.[15] This strict requirement for the proper length of the side chain is best explained by the distance between the residue(s) within the active site that binds the α-carboxyl of the monoglutamate and the residue(s) involved in modification of the γ-carboxyl during the catalytic step. The reactivity of **3** but not **4** and the complete inactivity of **2** strongly imply that it is the binding of the α-carboxyl on the *terminal* glutamate of an oligoglutamate to some residue in the active site that determines whether reaction at the γ-carboxyl occurs. One possible geometry to explain this behavior is shown in Figure 2. The fact that higher concentrations of **3** than of MeAPA-Glu-Glu are required to saturate the reaction might reflect differences in the three-dimensional structure of the two side chains, or may indicate a second role of the α-carboxyl of the first glutamate of an oligoglutamate in the enzymatic reaction. While these results certainly do not prove that only a single enzyme is involved in the synthesis of folyl or antifolyl oligoglutamates from monoglutamates in mammalian cells, they offer a logical explanation of how a single enzyme could perform this function.

Folyl polyglutamation has become a focus of the design of new antifolates in two ways: (1) as a new enzyme target[21] since FPGS activity is essential for the survival of

dividing cells[11,14,22] and (2) as an important determinant of the retention of antifols within tumor cells.[23,24] Our results (Figure 1) suggest that antifols lacking an α-carboxyl will not be polyglutamated in vivo and, hence, may be poorly retained in tumor cells. The results of this study also suggest that, if binding of the α-carboxyl to FPGS can be assumed to involve interaction with a nucleophilic basic amino acid, the introduction of a reactive substituent near, or in place of, the carboxyl group may be exploitable as a strategy for the design of FPGS inhibitors.

Experimental Section

A pure sample of MeAPA-Glu-Glu was a generous gift of Dr. John Montgomery (Southern Research Institute, Birmingham, AL). [3,4-^{3}H]-L-Glutamic acid was purchased from New England Nuclear (Boston, MA) and was purified before use by passage through a column of activated charcoal. 4-Aminobutyric acid, trimethylsilyl chloride, diphenylphosphoryl azide, and isobutyl chloroformate were purchased from Aldrich (Milwaukee, WI). α-Benzyl L-glutamate was obtained from Chemical Dynamics (South Plainfield, NJ). MeAPA and diethyl phosphorocyanidate were prepared and purified as previously described.[16] Benzene used in silylations and *N,N*-dimethylformamide (DMF) used in coupling reactions were dried over Linde 4A molecular sieves. Ion-exchange chromatography was carried out on Whatman DE-52 DEAE-cellulose, and TLC was performed on Avicel of silica gel plates with a fluorescent indicator (Analabs, North Haven, CT). Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Compounds for which melting points are not reported did not melt below 300 °C.

B6D2F1 female mice were obtained from Simonsen Laboratories (Gilroy, CA) and were fed standard laboratory chow ad libitum. Mice were sacrificed by cervical dislocation and the livers were perfused in situ with ice-cold 50 mM Hepes, pH 7.4, containing 0.25 M sucrose. Livers were removed, weighed, and homogenized in 2 volumes of this same buffer containing 20 mM α-thioglycerol using a motor-driven Teflon pestle. The homogenate was centrifuged for 1 h at 165000*g* and the supernate was brought to 30% saturation with $(NH_4)_2SO_4$ at 0 °C. The precipitated protein was dissolved in a minimal volume of homogenization buffer and insoluble material was removed by centrifugation. The supernatant protein was precipitated with 50% $(NH_4)_2SO_4$ and was stored until use in this form. $(NH_4)_2SO_4$ was removed prior to assay by Sephadex G-25 chromatography. Conjugase (i.e., folyloligoglutamate hydrolase) activity was measured under the conditions of the FPGS assay and under conditions optimal for conjugase by minor modifications of the method of Krumdieck and Baugh[25] as previously described.[9] Compounds were incubated with FPGS for 60 min at 37 °C in a mixture containing, in addition to a 4-aminofolyl substrate (0–1000 μM), 1 mM [^{3}H]-L-glutamic acid (4 mCi/mmol), 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 20 mM α-thioglycerol, and 200 mM Tris, pH 8.6, in a total volume of 0.25 mL. [^{3}H]Oligoglutamyl conjugates was isolated from incubation mixtures by adsorption onto acid-washed activated charcoal (Sigma Chemical Co., St Louis, MO) which had been treated with Dextran T-70 (25 mg/g charcoal; Pharmacia, Inc., Uppsala, Sweden). The charcoal was washed extensively to remove unreacted [^{3}H]-L-glutamate and the product was eluted from the charcoal with ethanolic ammonia. The characteristics of this assay have been described.[13] In the experiments reported here, there were 3.6 cpm/pmol of product and the FPGS typically had a specific activity of 1.2 nmol of product formed per hour per milligram of protein. Negligible charcoal-adsorbable products (<3% of control) were formed in incubations run in the absence of a folate analogue, ATP, or $MgCl_2$. Duplicate assays were performed for each condition per experiment and all experiments were performed at least twice. Data were analyzed by weighted nonlinear regression fitting to a rectangular hyperbola according to a standard statistical procedure for enzyme data.[20]

4-[*N*-(4-Amino-4-deoxy-*N*^{10}-methylpteroyl)amino]butyric Acid (MeAPA-Gaba, 2). To a stirred suspension of vacuum-dried 4-aminobutyric acid (206 mg, 4.4 mmol) in dry C_6H_6 (3 mL) at room temperature was added Et_3N (444 mg, 4.4 mmol) followed by Me_3SiCl (475 mg, 0.55 mmol). After overnight stirring and dilution with several volumes of hexane, the Et_3N·HCl was quickly filtered off, the filter cake was washed with hexane, and the combined filtrates were evaporated to an oil (439 mg, 89% yield) whose NMR spectrum was consistent with the expected *N,O*-bis(trimethylsilyl) derivative.

MeAPA (360 mg, 1.0 mmol) was added in small portions to a stirred solution of diethyl phosphorocyanidate (407 mg, 2.5 mmol) and Et_3N (253 mg, 2.5 mmol) in dry DMF (30 mL). After overnight stirring at room temperature, the silylated amino acid in a small volume of CCl_4 was added, followed by another 15 mL of DMF. The resulting cloudy mixture was left to stir overnight, the solvents were removed by rotary evaporation, and the residue was dissolved in dilute ammonia. Acidification with 10% AcOH and refrigeration at 4 °C caused a solid to form, which was collected, washed with H_2O, and dried under high vacuum, first in a lyophilization apparatus and then at 60 °C over P_2O_5; yield 418 mg (92%); R_f 0.4 (cellulose, pH 7.4 phosphate buffer); IR (KBr) ν 3370, 1710 (sh), 1600–1640 cm^{-1}. Anal. ($C_{19}H_{22}N_8O_3 \cdot 2.5H_2O$) C, H, N.

***N*-[4-[*N*-(4-Amino-4-deoxy-*N*^{10}-methylpteroyl)amino]-butyryl]-L-glutamic Acid (MeAPA-Gaba-Glu, 3).** To a suspension of vacuum-dried L-glutamic acid (0.441 g, 3.0 mmol) in C_6H_6 (10 mL) were added consecutively Et_3N (1 g, 10 mmol) and Me_3SiCl (1.08 g, 10 mmol). After being left to stir in a closed flask at room temperature overnight, the reaction mixture was diluted with hexane (10 mL) and the Et_3N·HCl was quickly removed by filtration. Evaporation of the filtrate afforded an oil (1.1 g, 97% yield) whose NMR spectrum indicated it to be the desired *N*-trimethylsilyl α,γ-bis(trimethylsilyl) ester.

A solution of **2** (319 mg, 0.7 mmol) in dry DMF (5 mL) was treated successively with Et_3N (140 mg, 1.4 mmol) and isobutyl chloroformate (191 mg, 1.4 mmol). After 30 min of stirring at room temperature, an additional 10% of each reagent was added, followed after 10 min by the DMF solution of the silylated amino acid (1.05 g, 3.0 mmol). The reaction was left to stir for 3 days, the solvent was evaporated under reduced pressure, and the residue was dissolved in dilute ammonia. The solution was acidified with 10% AcOH and refrigerated at 4 °C until a solid formed. The solid was collected, washed with H_2O, and passed through a DEAE-cellulose column with 3% NH_4HCO_3 as the eluent. Appropriate TLC-homogeneous fractions were pooled and freeze-dried to obtain a yellow solid (226 mg, 54% yield); R_f 0.70 (cellulose, pH 7.4 phosphate buffer); IR (KBr) ν 3330, 1615–1635 (amide C=O) cm^{-1}. Anal. ($C_{24}H_{29}N_9O_6 \cdot 3H_2O$) C, H, N.

Methotrexate γ-*N*-(3-Carboxypropyl)amide (MeAPA-Glu-Gaba, 4). A stirred suspension of vacuum-dried 4-aminobutyric acid (515 mg, 5.0 mmol) in ice-cold MeOH (15 mL) was treated dropwise over 20 min with $SOCl_2$ (5 mL) so that the internal temperature did not exceed 12 °C. After being left at room temperature overnight, the mixture was concentrated to dryness by rotary evaporation to obtain methyl 4-aminobutyrate hydrochloride (788 mg, quantitative yield); mp 122.5–123.0 °C (lit.[26] mp 121.5–122.5 °C).

To a stirred solution of the ester hydrochloride (84 mg, 0.55 mmol) and **6** (286 mg, 0.5 mmol)[18,19] in dry DMF (10 mL) at 0 °C were added successively diphenylphosphoryl azide (152 mg, 0.55 mmol) and Et_3N (162 mg, 1.6 mmol). After being kept at 0 °C for 2 h, the mixture was left at room temperature overnight. The solvent was removed by rotary evaporation, and the residue was taken up in $CHCl_3$. Extraction with dilute ammonia, evaporation of the $CHCl_3$ layer, and column chromatography of the residue on silica gel with 95:5 $CHCl_3$–MeOH as the eluent gave the diester, **7**, of **4** as a bright-yellow solid (197 mg, 61% yield); mp 97–104 °C; IR (KBr) ν 3330, 1730 (ester C=O), 1605 (amide

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C=O) cm^{-1}. Anal. ($C_{32}H_{37}N_9O_6 \cdot 0.5H_2O$) C, H, N.

$Ba(OH)_2 \cdot 8H_2O$ (126 mg, 0.4 mmol) was added to a stirred solution of the diester **7** (129 mg, 0.2 mmol) in 50% EtOH (10 mL). After 60 h, a solution of Na_2SO_4 (57 mg, 0.4 mmol) in H_2O was added, and the mixture was stirred vigorously for 5 min. The precipitate of $BaSO_4$ was removed by filtration and the filtrate was concentrated to a small volume by rotary evaporation and then freeze-dried. The product was desalted by passage through a DEAE-cellulose column, which was eluted first with a large volume of H_2O and then with 6% NH_4HCO_3. Appropriately pooled fractions of the latter eluent were freeze-dried to give a yellow solid (98 mg, 82% yield); R_f 0.80 (cellulose, pH 7.4 phosphate buffer); IR (KBr) ν 3340, 1615 cm^{-1}. Anal. ($C_{24}H_{29}N_9O_6 \cdot 3H_2O$) C, H, N.

Note Added in Proof: After this manuscript was submitted for publication, a symposium monograph appeared in which it was discussed that tetrahydrofolate and folate, i.e., 4-oxo, compounds analogous to **3** were excellent substrates for hog liver FPGS.[27] It was also stated in that symposium that compound **2** was not a substrate for rat liver enzyme.[28] Hence, the results shown in Figure 1 for mouse liver FPGS may be of more general applicability to FPGS from other mammalian sources.

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Registry No. **2**, 68352-96-5; **3**, 91425-22-8; **4**, 91425-23-9; **5**, 19741-14-1; **6**, 89106-05-8; **7**, 91425-24-0; MeAPA-Glu, 59-05-2; MeAPA-Glu-Glu, 41600-13-9; FPGS, 63363-84-8; 4-aminobutyric acid, 56-12-2; *N,O*-bis(trimethylsilyl)-4-aminobutyric acid, 39538-11-9; *N,O,O*-tris(trimethylsilyl)-L-glutamic acid, 15985-07-6; methyl 4-ammobutyrate hydrochloride, 13031-60-2; glutamic acid, 56-86-0.

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N-Allyl Analogues of Phencyclidine: Chemical Synthesis and Pharmacological Properties

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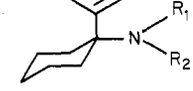
Several *N*-allyl derivatives of 1-phenylcyclohexylamine (PCA) were prepared, and their pharmacology was briefly characterized. The mono- and diallyl derivatives 4–7 had phencyclidine-like activities in mice but were less potent behaviorally than phencyclidine (PCP). None were PCP antagonists. In vitro these compounds were competitive inhibitors of butyrylcholinesterase (BChE) and protected against inhibition by DFP. In addition, these agents displaced tritiated *N*-methyl-4-piperidyl benzilate from mouse-brain homogenates and inhibited the effects of acetylcholine on isolated guinea pig ileum. None of these in vitro effects correlated with their PCP-like behavioral activity in vivo in mice.

N-(1-Phenylcyclohexyl)piperidine (phencyclidine, PCP, Sernyl, angel dust, peace pill) was originally developed as an analgesic anesthetic for humans but later withdrawn from clinical use because of its undesirable mental side effects. In recent years, it has become a drug of widespread abuse in the United States, resulting in severe incidents of psychoses including delirium, hallucinations, depression, coma, seizures, etc.

Although extensive pharmacological studies of PCP have been done, neither its mechanism of action is known nor has a specific pharmacological antagonist of PCP been found. Since it is known that the introduction of an allyl group into a pharmacologically active centrally acting compound may cause either an antagonism (e.g., naloxone) or potentiation/addition (e.g., barbiturates) of the original effects, we prepared and studied the pharmacology of a number of *N*-allyl derivatives (**4–7**) of 1-phenylcyclohexylamine (**1**, PCA) that are related to PCP. *N*-Allylnormetazocine and particularly the (+) enantiomer have been reported to produce PCP-like responses in laboratory animals.[1,2]



1, $R_1 = R_2 = H$
2, $R_1 = Me$; $R_2 = H$
3, $R_1 = Et$; $R_2 = H$
4, $R_1 = H$; $R_2 = CH_2{=}CHCH_2$
5, $R_1 = Me$; $R_2 = CH_2{=}CHCH_2$
6, $R_1 = Et$; $R_2 = CH_2{=}CHCH_2$
7, $R_1 = R_2 = CH_2{=}CHCH_2$

Results

Chemistry. The synthesis of the *N*-allyl derivatives was carried out according to Scheme I. (Alkylamino)cyclohexanecarbonitriles (**11**) were prepared from cyclohexanone (**8**), alkylamine hydrochloride (**9**), and sodium cyanide (**10**). Their reaction with phenyllithium yielded *N*-alkyl-1-phenylcyclohexylamines (**2**, **3**),[3,4] which with allyl

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