Mammalian Folylpoly-γ-glutamate Synthetase. 3. Specificity for Folate Analogues†

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Received January 31, 1986; Revised Manuscript Received September 25, 1986

ABSTRACT: A variety of folate analogues were synthesized to explore the specificity of the folate binding site of hog liver folylpolyglutamate synthetase and the requirements for catalysis. Modifications of the internal and terminal glutamate moieties of folate cause large drops in on rates and/or affinity for the protein. The only exceptions are glutamine, homocysteate, and ornithine analogues, indicating a less stringent specificity around the δ-carbon of glutamate. It is proposed that initial folate binding to the enzyme involves low-affinity interactions at a pterin and a glutamate site and that the first glutamate bound is the internal residue adjacent to the benzoyl group. Processive movement of the polyglutamate chain through the glutamate site and a possible conformational change in the protein when the terminal residue is bound would result in tight binding and would position the γ-carboxyl of the terminal glutamate in the correct position for catalysis. Steric limitations imposed on the internal glutamate residues that loop out and additional steric constraints imposed by binding of different pterin moieties would be expected to effect slight conformational changes in the protein and/or the terminal glutamate and would explain the decrease in on rate and catalytic rate with increased polyglutamate chain length, and the differential effect of one-carbon substitution on the catalytic rate with polyglutamate derivatives. The 4-amino substitution of folate increases the on rate for monoglutamate derivatives but severely impairs catalysis with diglutamate derivatives. Pteroylornithine derivatives are the first potent and specific inhibitors of folylpolyglutamate synthetase to be identified and may act as analogues of reaction intermediates. Other folate derivatives with tetrahedral chemistry replacing the peptide bond, such as pteroyl-γ-glutamyl-[ψ,CH_2-NH]-glutamate, retain affinity for the protein but are considerably less effective inhibitors than the ornithine derivatives.

In the preceding papers (Cichowicz & Shane, 1987a,b), the general properties, substrate specificity, and kinetic mechanism of hog liver folylpolyglutamate synthetase are described. The enzyme catalyzes the addition of glutamate moieties to a variety of folate mono- and polyglutamate substrates by a mechanism that is proposed to involve nucleophilic attack by the amino group of glutamate on a carboxyphosphate folate intermediate. Structure I (Figure 1) shows the proposed reaction intermediate.

In this report, the specificity of the enzyme for a variety of folate analogues has been assessed to determine the factors that are important in the binding of substrates and inhibitors to the folate binding site. Several classes of analogues have been synthesized and investigated. Pteroylmonoglutamate and -diglutamate analogues have been used to explore the enzyme specificity for the terminal and internal glutamates of folate. Compounds with tetrahedral geometry at the δ atom of the first glutamate moiety of the folate molecule were synthesized as potential analogues of reaction intermediates. These include Pte-homocysteine sulfonamide (II, Figure 1), Pte-ornithine (III), and PteGlu-[ψ,CH_2-NH]-Glu[1] (IV). A variety of pterin and pteroate analogues, synthesized as potential substrates or inhibitors of dihydrofolate synthetase, have also been investigated. Although mammalian cells lack dihydrofolate synthetase activity, dihydrofolate synthetase and folylpolyglutamate synthetase activities reside on a single polypeptide in some bacteria (Shane, 1980; Bognar et al., 1985). The proposed intermediates for the dihydrofolate synthetase reaction are shown in Figure 2 (V, VIII). Potential reaction intermediate analogues synthesized, with tetrahedral geometry at the carbonyl C of Pte, include 4-NH_2-Pte-sulfonamide (VI), 4-NH_2-Pte-[ψ,SO_2-NH]-Glu (VII, 4-NH_2-Pte-sulfanilyl-Glu), and 4-NH_2-Pte-phosphonate (IX).

† Supported in part by U.S. Public Health Service Grant CA 41991 and Research Career Development Award CA 01160 (B.S.) from the National Cancer Institute, Department of Health and Human Services, and NIH Predoctoral Fellowship ES 07067 (D.J.C.). A preliminary account of some of these studies was presented at the Seventh International Symposium on Pteridines and Folic Acid Derivatives at St. Andrews, Scotland, Sept 1982, and at a workship on Folyl and Antifolyl Polyglutamates at the Airlie Foundation, Airlie, VA, Nov 1984.

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[1] Abbreviations: Pte, pteroic acid; PteGlu, pteroylmonoglutamic acid (folic acid); $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylpoly-γ-glutamate, *n* indicating the number of glutamate moieties; *p*AbaGlu$_n$, *p*-aminobenzoylpoly-γ-glutamate; 4-NH_2-10-CH_3-PteGlu, methotrexate; 4-NH_2-Pte-carboxamide, 4-[[(2,4-diaminopteridin-6-yl)methyl]amino]-benzamide; 4-NH_2-Pte-sulfonamide, 4-[[(2,4-diaminopteridin-6-yl)-methyl]amino]benzenesulfonamide; 4-NH_2-Pte-sulfanilyl-Glu or Pte-[ψ,SO_2-NH]-Glu, *N*-[[4-[[(2,4-diaminopteridin-6-yl)methyl]amino]-phenyl]sulfonyl]glutamic acid; 4-NH_2-Pte-phosphonate, 4-[[(2,4-diaminopteridin-6-yl)methyl]amino]benzylphosphonate; pterin-6-carboxyglutamate, *N*-[(2-amino-4-hydroxypteridin-6-yl)carboxy]-L-glutamic acid; PteGlu-[ψ,CH_2-NH]-Glu, *N*-(*N*-pteroyl-1-amino-1-carboxybutan-4-yl)glutamic acid; DTBSF, 3-chloro-4-[4-[2-chloro-4-[4,6-diamino-2,2-dimethyl-*s*-triazin-1(2*H*)-yl]phenyl]butyl]benzenesulfonyl fluoride (NSC 127755); kainic acid, 2-carboxy-3-(carboxymethyl)-4-isopropenylpyrrolidine; homocysteine sulfonamide, 3-amino-3-carboxypropanesulfonamide; HPLC, high-performance liquid chromatography; CBZ, carbobenzoxy; TFA, trifluoroacetic acid; *p*Aba, *p*-aminobenzoyl.

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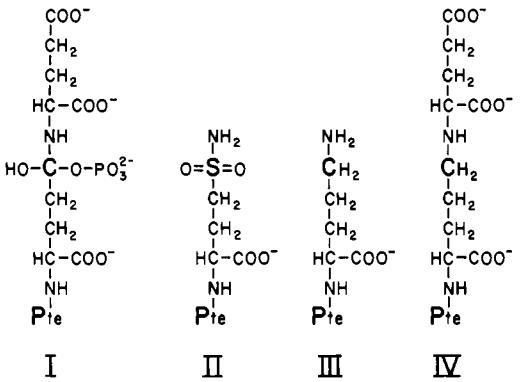

FIGURE 1: Potential reaction intermediate analogues of the folylpolyglutamate synthetase reaction. I, proposed transition-state intermediate for the reaction; II, pteroylhomocysteine sulfonamide; III, pteroylornithine; IV, Pte-γ-glutamyl-[ψ,CH_2-NH]-glutamate.

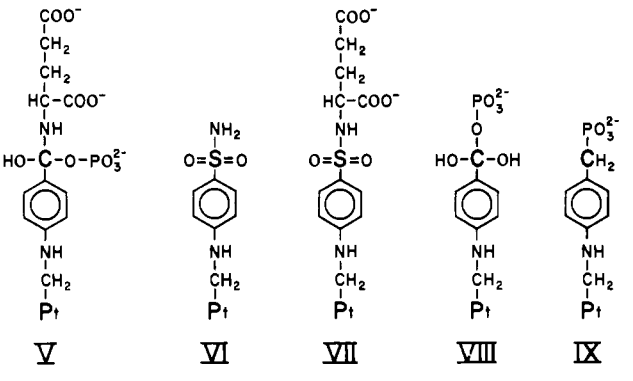

FIGURE 2: Potential reaction intermediate analogues of the dihydrofolate synthetase reaction. Pt, 2,4-diaminopteridin-6-yl or 2-amino-4-hydroxypteridin-6-yl; V and VIII, proposed intermediates for the reaction; VI, pteroylsulfonamide; VII, [ψ,SO_2-NH]aminopterin (4-aminopteroylsulfanilylglutamate); IX, 4-aminopteroylphosphonate.

Experimental Procedures

Materials. Substrates and reagents were synthesized or obtained from commercial sources as described in the preceding papers (Cichowicz & Shane, 1987a,b). 4-Amino-10-methyl-$PteGlu_{3,4}$, (6*RS*)-5-methyltetrahydrohomofolic acid, DTBSF, and 4-amino-6-(bromomethyl)pterin were obtained from Dr. J. A. R. Mead of the National Cancer Institute.

Synthesis of Folate Analogues. The synthesis of the folate analogues used in this study will be described in detail elsewhere (S. George and B. Shane, unpublished results). In brief, homocysteine sulfonamide was synthesized as described by Reisner (1956) and Mosher et al. (1958). *N*-Sulfanilylglutamate was synthesized by coupling *p*-nitrobenzenesulfonyl chloride to glutamate. *N*-(1-Amino-1-carboxybutan-4-yl)-glutamate (γ-Glu-[ψ,CH_2-NH]-Glu) was synthesized by forming a Schiff base between N^{α}-CBZ-ornithine and 2-oxoglutarate, reducing the complex, and deprotecting. Reduction of the Schiff base generates a chiral center at the C-2 of glutarate, and the presumed product is Glu-[ψ,CH_2-NH]-DL-Glu.

Pteroylmonoglutamate analogues were synthesized by coupling (trifluoroacetyl)pteroic acid to protected amino acid derivatives using isobutyl chloroformate (Plante et al., 1967) or carbonyldiimidazole as the coupling agent or by coupling 4-amino-6-(bromomethyl)pterin to 4-aminobenzoyl amino acid derivatives (Piper & Montgomery, 1977). The yield obtained with Pte-kainate was very low, reflecting that coupling was to a secondary amino group and that this group is sterically hindered.

Diglutamate analogues were synthesized by a solid phase procedure (Baugh et al., 1970). A major contaminant was found in the synthesis of Pte-β-L-Asp-L-Glu which was identified as Pte-α-L-Asp-L-Glu. These compounds were easily separated by ion-exchange chromatography. Significant lactam formation and subsequent chain migration were only observed with peptides containing internal aspartyl residues, and only trace amounts of chain migration were observed with peptides containing internal glutamyl residues.

4-Aminopterin analogues were synthesized by coupling 4-amino-6-(bromomethyl)pterin to the desired side chain. Pterin-6-carboxyglutamate was synthesized by coupling the TFA anhydride of pterin-6-carboxylate (Nair & Baugh, 1973) to glutamic acid diethyl ester.

All compounds were purified by ion-exchange, Sephadex gel, cellulose, and/or silica gel chromatography. Purification procedures were monitored by HPLC. The poor solubility of some of the folate analogues precluded their large-scale purification, and in some cases, poor yields were obtained after purification. In each case, the final product was homogeneous, as judged by HPLC. The identity of the final products was verified by NMR in 0.1 N NaOD using a Varian XL-400 or Bruker WM-300 instrument. In each case, with the exception of PteGlu-[ψ,CH_2-NH]-Glu, an unambiguous spectrum was obtained. The spectrum of the underivatized Glu-[ψ,CH_2-NH]-Glu dipeptide was consistent with the proposed structure. Similar chemical shifts were observed with the pteroyl derivative except that integration of the peaks suggested partial loss of the proton signals from two of the methylene peaks. The possibility that the terminal γ-carboxyl acylated the internal secondary amine to form the pyroglutamate derivative could not be completely excluded although was unlikely on the basis of the NMR spectrum. The reason for the apparent partial loss of signal remains unexplained.

Oxidized analogues were reduced to the dihydro, (6*RS*)-tetrahydro, and (6*S*)-tetrahydro forms by reduction with dithionite, with H_2 over Pt catalyst, and enzymatic reduction with dihydrofolate reductase, respectively, as described previously (Shane, 1980; Foo & Shane, 1982). The enzymatic reduction of folate analogues lacking an α-carboxyl group in the amino acid residue adjacent to the pteroyl moiety proceeded slowly (Bolin et al., 1982; Piper et al., 1982), necessitating the use of larger amounts of dihydrofolate reductase and longer incubation times.

Folylpolyglutamate Synthetase Assay. Enzyme activity was assayed by using the standard assay conditions [(6*RS*)-H_4PteGlu (40 μM), ATP (5 mM), [^{14}C]glutamate (250 μM), 2 h at 37 °C] described in the preceding paper (Cichowicz & Shane, 1987a). All assays utilized purified enzyme.

Folate analogue substrate specificities were compared by using the standard assay conditions modified by the replacement of H_4PteGlu with various concentrations of the folate derivative under study. The specificity of folate analogues for the folate binding site was assessed by adding analogue (usually 1 mM) to the standard assay mixture [containing 40 μM (6*RS*)-H_4PteGlu] and measuring the inhibition of labeled glutamate incorporation into folate products.

Kinetic parameters for folate analogues as substrates were measured by using a modified assay. Fixed substrates were ATP (1 mM) and [^{14}C]glutamate (2 mM, 2.5 mCi/mmol), and the assay time was reduced to 30 min or 1 h. Folate analogues were added at various concentrations. Kinetic parameters for folate analogue inhibitors were measured in a similar fashion except the assay mixtures contained various concentrations of (6*S*)-H_4PteGlu or PteGlu and a fixed concentration of the analogue inhibitor.

Table I: Pteroylmonoglutamate Analogues as Substrates for Folylpolyglutamate Synthetase[a]

analogue[b]	relative activity 10 μM	50 μM	100 μM	1 mM
(6*RS*)-H_4PteGlu	62	100	89	33
(6*RS*)-5-methyl-H_4-homofolate	1.3	*c*	10	31
9-methyl-PteGlu	0.8	*c*	6.3	20
PteGlu	5.7	17	33	47
homofolate	0.3	3.2	*c*	19
aminopterin	33	90	*c*	107
methotrexate	2.1	9.4	*c*	39

[a] The assay mixture was as described under Experimental Procedures and contained 5 mM ATP and 250 μM L-[^{14}C]glutamate. Folate analogues were added at 10, 50, 100, and 1000 μM, as indicated. Activities are expressed relative to that obtained with 50 μM (6*RS*)-H_4PteGlu as substrate. [b] No substrate activity detected with pterin-6-carboxyglutamate, Pte-L-aspartate, Pte-DL-2-aminoadipate, Pte-DL-2-aminopimelate, Pte-4-aminobutyrate, Pte-5-aminovalerate, Pte-DL-2-methyl-Glu, Pte-kainate, Pte-L-2-aminobutyrate, Pte-L-2-aminovalerate, Pte-DL-homocysteate, Pte-L-glutamine, Pte-L-ornithine, Pte-L-methionine sulfone, Pte-L-methionine sulfoxide, 4-NH_2-Pte-sulfanilyl-Glu, or 4-NH_2-H_2Pte-sulfanilyl-Glu. [c] Not determined.

Table II: Pteroylpolyglutamate Analogues as Substrates for Folylpolyglutamate Synthetase[a]

analogue	relative activity 10 μM	50 μM	100 μM	1 mM
PteGlu	*b*	*b*	33	42
$PteGlu_2$	*b*	45	116	120
4-NH_2-10-CH_3-$PteGlu_3$	*b*	0	*b*	*b*
4-NH_2-10-CH_3-$PteGlu_4$	*b*	0	*b*	*b*
Pte-Gly-Glu	*b*	0	0.4	1.7
Pte-β-Asp-Glu	*b*	0	*b*	*b*
Pte-β-Ala-Glu	*b*	*b*	3.2	7.0
Pte-(4-aminobutyryl)-Glu	*b*	4.0	21	89
Pte-(5-aminovaleryl)-Glu	*b*	*b*	2.3	7.2
PteGlu-[ψ,CH_2-NH]-Glu	*b*	*b*	0	*b*
(6*RS*)-H_4PteGlu	69	100	89	33
(6*RS*)-$H_4PteGlu_2$	86	127	139	127
(6*RS*)-H_4Pte-α-Asp-Glu	*b*	*b*	0.5	*b*
(6*RS*)-H_4Pte-β-Asp-Glu	*b*	*b*	3.9	*b*
(6*RS*)-H_4Pte-γ-Glu-Asp	*b*	*b*	0	*b*
(6*RS*)-H_4Pte-Gly-Glu	*b*	*b*	10	*b*
(6*RS*)-H_4Pte-(4-aminobutyryl)-Glu	*b*	*b*	168	*b*

[a] The assay mixture was as described under Experimental Procedures and contained 5 mM ATP and 250 μM L-[^{14}C]glutamate. Folate analogues were added at 10, 50, 100, and 1000 μM, as indicated. Activities are expressed relative to that obtained with 50 μM (6*RS*)-H_4PteGlu as substrate. [b] Not determined.

Table III: Reduced Analogues as Substrates for Folylpolyglutamate Synthetase[a]

X[b]	(6*S*)-H_4Pte-X	(6*R*)-5,10-methylene-H_4Pte-X	(6*R*)-10-formyl-H_4Pte-X
Glu[c]	100	10	26
Glu	146	29	45
Glu_2	63	27	20
L-β-Asp-Glu	9.9	5.1	0
Gly-Glu	0.7	0	0
β-Ala-Glu	21	*d*	*d*
(4-aminobutyryl)-Glu	51	48	15
(5-aminovaleryl)-Glu	6.9	*d*	*d*
homofolate	6.6	*d*	*d*

[a] The assay mixtures were as described in the legend to Table II except the indicated enzymatically reduced folate analogues (50 μM) were used as substrates. Activities are expressed relative to that obtained with 50 μM (6*RS*)-H_4PteGlu as substrate. [b] No activity detected with L-Asp, DL-2-aminoadipate, DL-2-aminopimelate, 4-aminobutyrate, or 5-aminovalerate derivatives. [c] 6*RS* derivative. [d] Not determined.

Table IV: Kinetic Constants of Folate Analogues for Folylpolyglutamate Synthetase[a]

analogue	K_m (μM)	V_{max}(rel)[b]	V_{max}/K_m-(rel)[b]
PteGlu	93	61	5.1
$PteGlu_2$	62	60	7.4
aminopterin	21	88	32
methotrexate	138	62	3.4
Pte-β-Ala-Glu	690	13	0.14
Pte-(4-aminobutyryl)-Glu	810	50	0.47
Pte-(5-aminovaleryl)-Glu	1090	21	0.15
H_2PteGlu	5.0	101	156
$H_2PteGlu_2$	2.6	51	152
H_2-aminopterin	7.3	80	84
H_2Pte-β-Ala-Glu	76	31	3.1
H_2Pte-(4-aminobutyryl)-Glu	12	39	25
H_2Pte-(5-aminovaleryl)-Glu	22	24	8.3
(6*S*)-H_4PteGlu	7.7	100	100
(6*S*)-$H_4PteGlu_2$	3.4	45	102
(6*S*)-H_4Pte-β-Ala-Glu	160	51	2.5
(6*S*)-H_4Pte-(4-aminobutyryl)-Glu	74	68	7.1
(6*S*)-H_4Pte-(5-aminovaleryl)-Glu	163	20	1.0
(6*RS*)-5-methyl-H_4-homofolate	444	37	0.64

[a] Enzyme activity was assayed as described under Experimental Procedures using 1 mM ATP and 2 mM L-[^{14}C]glutamate as the fixed substrates and various concentrations of the indicated folate analogue as the variable substrate. [b] Values are relative to those obtained with (6*S*)-H_4PteGlu as the substrate. For V_{max}(rel), 100 equals 123 μmol h^{-1} mg^{-1}.

Results

Specificity for Pteroylmonoglutamate Analogues. The specificity of the enzyme for pteroylmonoglutamate analogues was initially assessed by using subsaturating levels of glutamate (standard assay, 250 μM, Tables I and III). Aminopterin was a better substrate for the enzyme than PteGlu, with activity approaching that observed with H_4PteGlu, while methotrexate, 9-methyl-PteGlu, homofolate, H_4-homofolate, and 5-methyl-H_4-homofolate were poorer substrates with high apparent K_m's (Tables I and III). Pterin-6-carboxyglutamate, a folate analogue that lacks the *p*Aba moiety, was not a substrate (Table I).

Essentially all modifications of the glutamate moiety of PteGlu led to loss of substrate activity (Table I). These included the aspartate, aminoadipate, and aminopimelate homologues and their α- and ω-decarboxylation products and the 2-methylglutamate analogue. Similar results were found with the tetrahydro forms of these analogues (Table III). No activity was observed with the homocysteate and methionione sulfone derivatives (Table I).

Kinetic parameters for the analogues were evaluated by using near-saturating glutamate concentrations (2 mM, Table IV). The substrate effectiveness of the different compounds was assessed by comparing V_{max}/K_m ratios to that obtained with (6*S*)-H_4PteGlu. The affinities of compounds which lacked substrate activity were assessed by K_i values (Table V).

Aminopterin was about one-third as effective a substrate as H_4PteGlu with a similar V_{max} and a 3-fold higher K_m. It was considerably more effective than PteGlu while methotrexate was less effective than PteGlu (Table IV). Dihydroaminopterin was as good a substrate as H_4PteGlu although somewhat less effective than H_2PteGlu. 5-Methyl-H_4-homofolate was a very poor substrate.

In most cases, the modifications of the glutamate moiety of PteGlu that led to loss of substrate activity (Table I) were also reflected by a loss of affinity for the enzyme. K_i values in excess of 1 mM were found for the aspartate, aminopimelate, and aminoadipate derivatives and their decarboxylation products and for Pte-4-aminobutyrate and values in excess of 100 μM for their (6*S*)-tetrahydro forms. The major

Table V: Kinetic Constants of Folate Analogue Inhibitors of Folylpolyglutamate Synthetase[a]

analogue (X)	K_i (μM) X	H_2X	(6S)-H_4X
9-methyl-PteGlu	650	b	b
$PteGluNH_2$	113	22	10
PteGlu-diamide	>10000	b	b
Pte-DL-2-methylglutamate	>5000	b	>100
Pte-L-2-aminobutyrate	b	b	68
Pte-kainate	164	b	b
Pte-DL-homocysteate	278	28	43
Pte-DL-homocysteine sulfonamide	342	b	72
Pte-Met-sulfone	b	b	>100
Pte-ornithine	5.9	0.2	0.2
PteGlu-[ψ,CH_2-NH]-Glu	32	b	b
4-amino-Pte-sulfanilyl-Glu	42	b	b
4-amino-Pte-phosphonate	~700	b	b

[a] Enzyme activity was assayed as described under Experimental Procedures using 1 mM ATP and 2 mM L-[^{14}C]glutamate as the fixed substrates and various concentrations of PteGlu or (6S)-H_4PteGlu as the variable substrate. K_i values were assessed by adding fixed concentrations of the indicated analogues to the assay mixtures. [b] Not determined.

exception was Pte-glutamine. The oxidized and reduced forms of this compound displayed similar K_i values to the K_m values obtained with PteGlu and its reduced forms (Table V). However, amidation of the α-carboxyl of glutamate, as shown by the diamide derivative, or introduction of a methyl group at the 2-carbon, led to complete loss of affinity (Table V). Pte-2-aminobutyrate retained low affinity for the protein while Pte-2-aminovalerate had no apparent affinity. Pte-kainate, which contains a conformationally restricted glutamate moiety, had similar affinity to $PteGluNH_2$ (Table V) but lacked detectable substrate activity (Table I).

Pte-homocysteate, which lacked substrate activity, had reduced affinity compared to $PteGluNH_2$ while Pte-methionine sulfone had little affinity for the protein (K_i > 1 mM). Pterin-6-carboxyglutamate and pterin-6-(hydroxymethyl)pyrophosphate were not inhibitors of the reaction (K_i > 1 mM).

DTBSF, a potent inhibitor and covalent inactivator of dihydrofolate reductase (Kumar et al., 1981), was an effective inhibitor of folylpolyglutamate synthetase provided high concentrations (1 mM) were added to the 2-h assay mixture. No inhibition was observed in short-incubation kinetic analyses using 100 μM DTBSF, suggesting that this compound has poor affinity for the protein but causes a slow covalent inactivation.

Specificity for Pteroylpolyglutamate Analogues. The enzyme specificity for a variety of pteroylpolyglutamate analogues is shown in Tables II and III. No activity was detected with the tri- and tetraglutamate analogues of methotrexate (Table II). Substitution of the terminal glutamate of $PteGlu_2$ with aspartate led to complete loss of activity (Pte-γ-Glu-Asp, Table II).

A variety of pteroyldiglutamate analogues were synthesized to explore the specificity for the internal glutamate residues. Low levels of activity were observed with oxidized or reduced Pte-Gly-Glu and Pte-Asp-Glu (Tables II and III). The decarboxylation derivative of the internal glutamate of $PteGlu_2$, Pte-(4-aminobutyryl)-Glu, was an effective substrate, both in its oxidized and in its reduced forms (Tables II and III). The equivalent decarboxylation products of the $PteGlu_2$ homologues Pte-Asp-Glu and Pte-aminoadipyl-Glu, viz., Pte-β-Ala-Glu and Pte-5-(aminovaleryl)-Glu, were also substrates but not as effective as Pte-(4-aminobutyryl)-Glu (Tables II and III). However, decarboxylation of Pte-Asp-Glu did convert this compound into a much more effective substrate. Kinetic constants for the pteroyldiglutamate analogues are shown in Table IV.

Specificity for Potential Reaction Intermediate Analogues. Several compounds with tetrahedral chemistry replacing the peptide bond between pteroate and the first glutamate residue of folate and between the two glutamate residues of pteroyldiglutamate were synthesized. It was speculated that effective binding of such compounds would require compounds with a terminal glutamate residue. 4-NH_2-Pte-sulfanilyl-Glu ([ψ,SO_2-NH]aminopterin), which was synthesized as a potential inhibitor of dihydrofolate synthetase, lacked substrate activity for folylpolyglutamate synthetase (Table I) but was a good inhibitor of the reaction with about 4-fold reduced affinity compared to aminopterin (Table V). PteGlu-[ψ,-CH_2-NH]-DL-Glu also lacked substrate activity (Table II) but retained good affinity for the enzyme. The presumed affinity of the L isomer of this compound (16 μM, Table V, assuming the D isomer lacks affinity) is significantly lower than the K_m for $PteGlu_2$ (62 μM) when measured under the same assay conditions.

Pte-ornithine was a potent inhibitor of the reaction. This compound was only synthesized and tested as a parent compound of PteGlu-[ψ,CH_2-NH]-Glu and was not expected to bind to the protein as it lacked a 4-carboxyl or 4-sulfonyl group. Pte-ornithine lacked substrate activity (Table I) but bound to the protein with a K_i of about 6 μM (Table V). Reduced forms of this compound were very potent inhibitors of the reaction with K_i values of about 0.2 μM (Table V).

The affinity of Pte-homocysteine sulfonamide, a $PteGluNH_2$ analogue with tetrahedral chemistry at the δ atom, was slightly less than that observed with Pte-homocysteate and about 3-fold lower than that of $PteGluNH_2$ (Table V). Potential pterin and pteroate analogue inhibitors of dihydrofolate synthetase, such as 4-amino-Pte-phosphonate, 4-amino-Pte-carboxamide, and 4-amino-Pte-sulfonamide, had little, if any, affinity for folylpolyglutamate synthetase.

Discussion

The specificity of the folate binding site of hog liver folylpolyglutamate synthetase and the requirements for effective substrate activity were further explored by investigating the affinity and/or substrate activity of a variety of folate analogues. Although the affinities of substrates were not measured directly, the on rates for these analogues can be assessed by k_{cat}/K_m ratios (Cichowicz & Shane, 1987b). The K_i values for analogues that lack substrate activity represent dissociation constants provided these analogues are dead-end inhibitors. Some compounds that lack apparent affinity and substrate activity may be very poor substrates with very high K_m values. Similarly, low apparent affinities with poor substrates, assessed by competition studies, are also indicative of high K_m values. In both these cases, the high K_m's would indicate very low on rates for the analogues and, indirectly, low affinities (k_{off}/k_{on}).

Specificity for the Pteroyl Moiety. Replacement of the 4-oxo group of PteGlu with an amino group increases the on rate about 5-fold without significantly affecting k_{cat} while 10-methyl substitution (methotrexate) causes a large decrease in the on rate. Although the on rate for H_2-aminopterin is about 3-fold higher than for aminopterin, the 4-amino substitution at the dihydro level adversely affects the on rate. Similar substrate specificities have been reported for other mammalian folylpolyglutamate synthetases (McGuire et al., 1980; Bognar et al., 1983; Schoo et al., 1985; Moran & Colman, 1984; Moran et al., 1985a). 7-Hydroxylation of aminopterin and methotrexate slightly lowers the apparent K_m of these substrates (McGuire et al., 1984; Moran et al., 1985a; Schoo et al., 1985), but this modification appears to decrease k_{cat} rather than affect the on rate.

No substrate activity was observed with tri- and tetraglutamate derivatives of methotrexate at the concentrations tested, and metabolism studies indicate that the diglutamate and the equivalent aminopterin derivative are very poor substrates (Cook et al., 1987). The diglutamate of methotrexate is a fairly good substrate for the mouse liver enzyme (Moran et al., 1984) but a poor substrate for the beef liver enzyme (Schoo et al., 1985). The triglutamate is essentially inactive as a substrate for the rat liver (McGuire et al., 1983a) and beef liver enzymes. However, long glutamate chain length methotrexate derivatives retain apparent affinity for the beef liver enzyme (Schoo et al., 1985). Although some differences in specificity for methotrexate derivatives occur with different mammalian enzymes, the 4-amino substitution enhances binding to the protein but hinders catalysis, at least with polyglutamate derivatives.

Some 5,8-dideazafolate derivatives are very good substrates for the enzyme with activities similar to (6*S*)-H_4PteGlu (McGuire et al., 1983b; Hynes et al., 1986), indicating that a pyrazine ring is not required for substrate binding or catalysis. Although 5-substitution of folates appears to adversely affect on rates (Cichowicz & Shane, 1987b), 5-chloro substitution of dideazafolates causes a large increase in the on rate (Hynes et al., 1986).

Methylation at the 9-position, extending the C-9–N-10 bond by an additional methylene group (homofolate), or replacement of N-10 with a methylene group (10-deazaaminopterin) causes large decreases in on rates (Nair et al., 1985; Moran et al., 1985a). Dideazaisofolate analogues, in which the C-9 and N-10 atoms are transposed, also have decreased on rates (Hynes et al., 1986) while halogenation of the *p*Aba ring (dichloromethotrexate; Moran et al., 1985a) appears to increase the on rate. Deletion of the *p*Aba moiety (pterin-6-carboxyglutamate) eliminates substrate activity and apparent affinity. In these studies, loss of affinity refers to K_i values in excess of 1 mM. Pterin derivatives in general seem to have very weak affinity for the protein with K_i values in the range of 1–10 mM. However, it is not possible to get more accurate values due to the poor solubility of these compounds.

These data further indicate that binding of folate substrates and analogues to the protein is dependent on the state of reduction and substitution of the pterin moiety and suggest that the *p*Aba moiety also interacts with the protein.

Removal of the pterin moiety (*p*AbaGlu) appears to result in complete loss of affinity and substrate activity for the hog liver and all bacterial synthetases. However, recent studies indicate that *p*AbaGlu is a substrate for the *Corynebacterium* enzyme, a protein with a tighter specificity for pteroylmonoglutamate substrates than the mammalian enzyme. The k_{cat} with *p*AbaGlu is similar to that obtained with the preferred monoglutamate substrate (H_4PteGlu) while the on rate is decreased about 5 orders of magnitude (unpublished data). The pterin moiety is required for effective binding and not for catalysis.

Specificity for Pteroylmonoglutamate Analogues. The specificity for the L-glutamate moiety of pteroylmonoglutamates is similar to, but not identical with, that found for the L-glutamate binding site (Cichowicz & Shane, 1987b). Decarboxylation of the α-carboxyl group of PteGlu, methylation at C-2, amidation of the α-carboxyl group, and substitution with aspartate, 2-aminoadipate, or 2-aminopimelate, or the decarboxylation products of these homologues, lead to loss of detectable substrate activity and affinity for the folate binding site. H_4Pte-aminopimelate is a very poor substrate for the *Corynebacterium* enzyme (unpublished data), and the equivalent 2-aminopimelate derivative of methotrexate is a very poor substrate for the mouse liver enzyme (Moran et al., 1985a) with a greatly increased K_m. Although no products were detected with the hog liver enzyme using oxidized and reduced Pte-2-aminopimelate derivatives, it is possible that they are poor substrates. The lack of apparent affinity would then indicate very high K_m values and consequently greatly reduced on rates and would suggest a large reduction in affinity. Substitution of the γ-carboxyl group of folate with a sulfonyl group eliminates substrate activity, but the compound retains affinity for the enzyme with K_i values about 2–4-fold higher than the K_m for pteroylmonoglutamate, depending on the state of reduction of the molecule. The equivalent homocysteate analogues of aminopterin and methotrexate have reasonable affinity for the mouse liver enzyme (Rosowsky et al., 1984a,b).

Although this specificity is similar to that of the glutamate binding site, other modifications of the glutamate moiety of folate reveal significant differences. Pte$GluNH_2$ retains good affinity for the enzyme, and the affinity for Pte-ornithine is about 30-fold greater, while the parent amino acids have no apparent affinity for the glutamate site. Kainate, a hindered glutamate analogue with the carbon chain in an extended configuration, lacks affinity for the glutamate site while Pte-kainate retains good affinity for the folate binding site, suggesting that different conformations of glutamate may bind to the folate and glutamate sites. Similarly, replacement of the γ-carboxyl of methotrexate with a phosphate group and the cysteate analogue retains affinity for the mouse liver enzyme (Moran et al., 1985b) while γ-fluoromethotrexate is a poor substrate for the rat liver enzyme (Galivan et al., 1985), although 4-fluoroglutamate is an effective alternate substrate for glutamate. Apart from the obvious difference that N-acylation of glutamate with pteroate is allowed, the glutamate binding portion of the folate binding site differs from the glutamate binding site in that the specificity around the δ-carbon is less stringent. Some interaction around the δ-carbon is still evident as the affinity of Pte-2-aminobutyrate is reduced and Pte-2-aminovalerate lacks measurable affinity. The glutamate binding site, which presumably forms on the protein after the addition of the folate substrate, does not appear to be a subset of the folate binding site. This specificity for the glutamate residue of pteroylmonoglutamates is similar to that reported for bacterial (Cichowicz et al., 1981; Shane, 1982; Shane & Cichowicz, 1983) and partially purified mammalian folylpolyglutamate synthetases (Bognar et al., 1983; McGuire et al., 1983b; Moran et al., 1984, 1985a).

Although binding of folate substrates has been interpreted in terms of on rates rather than affinities, the 20–30-fold difference in on rates between oxidized and reduced PteGlu is similar to the 10–30-fold difference in affinity between oxidized and reduced folate analogue inhibitors. This indicates that the on rates are the major factor in determining the differing affinities of oxidized and reduced derivatives and would indicate little, if any, differences in off rates of these compounds.

Specificity for Pteroyldiglutamate Analogues. Replacement of the terminal glutamate of $PteGlu_2$ with aspartate leads to loss of substrate activity and affinity for the protein. Similarly, replacement of the terminal glutamate of 4-amino-10-methyl-$PteGlu_2$ with 4-aminobutyrate eliminates substrate activity for the mouse liver enzyme (Moran et al., 1984). In the limited studies carried out, the specificity noted for the glutamate moiety of pteroylmonoglutamates holds for the terminal glutamate residue of pteroyldiglutamates.

Decarboxylation of the internal glutamate residue of $PteGlu_2$ does not significantly affect k_{cat}, but the on rates for Pte-(4-

aminobutyryl)glutamate derivatives are reduced 6–15-fold depending on the state of reduction of the molecule. The on rates for the β-alanine and 5-aminovaleryl homologues of Pte-(4-aminobutyryl)glutamate are reduced 30–100-fold while k_{cat} values are reduced only about 2-fold. On rates are reduced even further for Pte-Glycylglutamate and Pte-Asp-Glu derivatives. The binding specificity for the internal glutamate of pteroyldiglutamates is similar to, if not identical with, the terminal residue although modified internal glutamate analogues still retain substrate activity. Increases in K_m without changes in k_{cat} with Pte-(4-aminobutyryl)glutamate derivatives (Bognar et al., 1983) and the equivalent methotrexate derivative (Moran et al., 1984) have been previously reported.

The lack of affinity of a variety of glutamate analogues for the glutamate binding site (Cichowicz & Shane, 1987b), which also indicates their lack of affinity for the folate binding site, coupled with the low, but detectable, affinity of pterin derivatives, and the even lower affinity of *p*AbaGlu for folylpolyglutamate synthetase, suggests that tight binding of folates to the enzyme reflects a cooperative effect resulting from two low-affinity events, the initial binding of the pterin or pteroate moiety followed by the binding of a glutamate residue. High-affinity binding would then result from a conformational change in the protein, suggested by the low on rates for folate substrates (Cichowicz & Shane, 1987b).

It has been proposed that binding of folylpolyglutamates involves the pterin and terminal glutamate moieties (Cichowicz et al., 1981) and that internal glutamate residues loop out of the active site (Moran et al., 1984). However, the similar binding specificity for the internal and terminal glutamate residues of pteroyldiglutamate analogues is not consistent with this mechanism. This specificity is best explained by the initial binding of the pterin moiety and the internal glutamate residue of pteroyldiglutamates. Channeling of glutamate residues through this single, low-affinity glutamate binding site would occur until the terminal glutamate residue is bound. The conformational change required for tight binding would not occur when an internal glutamate residue is bound due to interference by the nonbound C-terminal residues. This mechanism is consistent with the retention of affinity by $PteGluNH_2$, which can be considered an analogue of pteroate with an internal glutamate residue and an unsubstituted C terminal. The identical on rates for some pteroylmono- and diglutamates (Cichowicz & Shane, 1987b) would suggest that processive movement from the internal glutamate to the terminal residue is not rate limiting with these compounds. Decreased on rates with longer chain length derivatives might result from random movement of the polyglutamate chain and the consequent decreased likelihood of the terminal residue being positioned in the site, or by the increased difficulty in moving the internal residues out of the site, due to steric effects. This mechanism would also presumably position the terminal glutamate residue in the appropriate position for catalysis (see below).

Requirements for Catalysis. Catalysis requires placement of the γ-carboxyl group of the terminal glutamate residue of folate in the correct position for, presumably, phosphorylation and amidation. Nearly all modifications of the glutamate moiety of pteroylmonoglutamate result in loss of catalytic activity but also result in loss of affinity for the folate binding site. Consequently, it is not possible to determine which, if any, of these modifications affect the catalytic process per se. The only exception is the kainate derivative, which retains some affinity but lacks catalytic activity, suggesting that different conformations of the glutamate residue of folate are required for binding and catalysis. Further elaboration of the conformations required for binding and/or catalysis, using hindered glutamate analogues such as cycloglutamate, was not carried out as 2-methylglutamate and its pteroyl derivative lack substrate activity and any detectable affinity for the glutamate and folate binding sites, respectively. The lack of substrate activity of pteroylmono- and diglutamate derivatives in which the α-carboxyl of the terminal glutamate residue is removed may reflect that binding of the α-carboxyl group to some residue in the active site determines whether catalysis at the γ-carboxyl occurs (Moran et al., 1984), although this specificity could be due to lack of affinity for the enzyme, rather than a requirement for catalysis.

Unlike other enzymes that possess affinity for folylpolyglutamates with a wide range of glutamate chain lengths, the synthetase is required to position the γ-carboxyl of the terminal glutamate in the active site for catalysis to occur. Pteroylmonoglutamates have the optimal configuration for catalysis, and extension of the glutamate chain causes increased difficulty in positioning the terminal glutamate residue in the active site (Cichowicz & Shane, 1987b). The different effects of modifications of the pterin moiety on k_{cat}'s of folate derivatives as the glutamate chain is extended strongly suggest that the pterin moiety remains bound to a specific site throughout catalysis. Although the pterin moiety is not absolutely required for catalysis (viz., *p*AbaGlu), if the pterin moiety is not bound differences in catalytic efficiency between various substituted polyglutamate derivatives should be decreased rather than enhanced as the glutamate chain is extended. If the pterin moiety and terminal glutamate are bound at specific sites during catalysis, the two sites would have to be in fairly close proximity to each other to allow catalysis with pteroylmonoglutamates. The ability to accommodate the internal glutamate residues of polyglutamate substrates by a looping-out mechanism would be expected to be limited by steric considerations. Slight changes in conformation of the protein and/or the positioning of the terminal glutamate in the active site would be expected in response to accommodation of the internal residues, and this would explain the decreased k_{cat} with extension of the polyglutamate chain.

Substitution and/or reduction of the pterin moiety of folate affects the aromaticity of the pteridine ring system, the relationship of the *p*Aba ring to the pteridine ring, and the relationship between the first glutamate residue and the pterin moiety. Steric limitations imposed on the position of internal glutamate residues that loop out would be expected to effect slightly different conformational changes in the protein depending on the pterin moiety that is bound, and differences in conformation with different pterin moieties may become more extreme with increases in chain length due to greater steric limitations on the looped-out chain. This would explain the large differences in catalytic effectiveness between different pteroylpolyglutamates but relatively minor differences between the equivalent pteroylmonoglutamates.

It is not possible to distinguish whether the lowered catalytic activity with polyglutamate substrates is due to an impairment in phosphorylation and/or amidation. Although the hog liver enzyme can be purified with reasonable recovery of activity, the low amounts of purified protein obtained preclude any detailed physical studies on the catalytic mechanism. Physical studies, aimed at elucidating the catalytic mechanism, are currently under way using the *Escherichia coli* protein, which has been obtained in large yield by using recombinant DNA techniques (Bognar et al., 1985).

Potential Reaction Intermediate Analogues. A variety of potential analogues of reaction intermediates of the folylpolyglutamate synthetase and dihydrofolate synthetase reac-

tions were synthesized and tested as inhibitors of the hog liver enzyme.

Homocysteine sulfonamide is a potent transition-state inhibitor of both glutamine synthetase (Meek & Villafranca, 1980) and glutamate synthase (Masters & Meister, 1982). The equivalent pteroyl analogue is only a weak inhibitor of folylpolyglutamate synthetase, with slightly lower affinity than Pte-homocysteate, and the compound appears to act as a substrate analogue rather than an analogue of the transition state. Methionine sulfone is an effective inhibitor of glutamine synthetase (Rowe & Meister, 1973). The analogous pteroyl derivative has no detectable affinity for the hog liver enzyme. Pterin and pteroate analogues exhibit little affinity for the enzyme, presumably reflecting the absence of a terminal glutamate residue. However, DTBSF, a pterin derivative with low affinity for the protein (K_i about 1 mM), slowly inactivates the hog liver enzyme.

4-Amino-Pte-sulfanilyl-Glu ([ψ,SO_2-NH]aminopterin), a compound with tetrahedral chemistry around its peptide bond and possessing a terminal glutamate residue, has similar affinity to aminopterin but lacks substrate activity. Similarly, PteGlu-[ψ,CH_2-NH]-Glu, a $PteGlu_2$ analogue with tetrahedral chemistry around the peptide bond, is not a substrate although it has higher apparent affinity for the enzyme than $PteGlu_2$. The lack of substrate activity with these compounds suggests they may be recognized as reaction intermediates or poor analogues of the transition state. However, they may bind as substrates, and the distorted chemistry around the peptide bond may prevent the correct positioning of their terminal glutamate residues for catalysis. [ψ,CH_2-NH]Aminopterin lacks substrate activity for the mouse liver enzyme but also lacks appreciable affinity (Moran et al., 1985a).

Unexpectedly, Pte-ornithine derivatives have very high affinity for the folate binding site, with K_i values about 30-fold lower than the K_m values of the equivalent PteGlu derivatives, and are the first potent specific inhibitors of folylpolyglutamate synthetase to be identified. Although K_{is} values for the folate substrates have not been determined, the similar K_m and K_{is} values obtained for aminopterin suggest that Pte-ornithine derivatives bind much tighter than the equivalent folate substrates. Following a preliminary report of the inhibitory action of Pte-ornithine derivatives (Cichowicz et al., 1985), other investigators (McGuire et al., 1986; Rosowsky et al., 1986) demonstrated that the equivalent ornithine analogues of aminopterin and methotrexate are potent inhibitors of folylpolyglutamate synthetases from rat, mouse, and human sources and that substitution of ornithine with other 2,ω-diaminoalkanoic acids, such as lysine and 2,4-diaminobutanoate, leads to a large drop in affinity for the enzyme. Although the reason for the high affinity of this compound for the enzyme is not understood, the potent inhibition observed with enzymes from different sources suggests that the ω-amino group is interacting with a conserved functional group of the enzyme that is presumably essential for substrate binding or catalysis.

It is possible that the compound is acting as a bisubstrate analogue with the ω-amino group occupying the position of the amino group of the entering glutamate, or it is recognized as a reaction intermediate. Nucleophilic attack by the free amine of glutamate on a folyl acylphosphate intermediate would be expected to result in protonation of the entering amino nitrogen. Catalysis would require removal of a proton from the amine and donation of a proton to the phosphate leaving group. The protonated ω-amino group of Pte-ornithine may be interacting with a base on the enzyme that stabilizes the transition state and/or abstracts the proton. If either of these possibilities are the case, PteGlu-[ψ,CH_2-NH]-Glu, which is also probably protonated on the secondary amine, would be expected to act as a better bisubstrate or transition-state analogue than Pte-ornithine. However, space-filling models of the proposed transition state indicate a distorted rigid conformation, due to the bulky phosphate group and possibly charge repulsion between the phosphate group and the α-carboxyl group of the entering glutamate, which is difficult for PteGlu-[ψ,CH_2-NH]-Glu to assume.

A third possibility, that Pte-ornithine is phosphorylated and its high affinity is due to a phosphoamidate species, is under investigation. This would explain the lowered affinity of PteGlu-[ψ,CH_2-NH]-Glu. 4-Amino-10-methyl-Pte-(4-aminobutyryl)phosphonate, an analogue of the proposed acyl phosphate intermediate formed during catalysis, lacks affinity for the mouse liver enzyme (Tang & Coward, 1983a,b). However, the lack of affinity is probably due to the lack of a α-carboxyl group.

ACKNOWLEDGMENTS

We thank Dr. Lou-sing Kan (Division of Biophysics, The Johns Hopkins University) and Joseph Cousins (Department of Chemistry, The Johns Hopkins University) for their assistance in the NMR analyses and Drs. John McGuire (Yale University Medical School) and Richard Moran (University of Southern California) for making available prepublication information on their studies with ornithine derivatives of aminopterin and methotrexate. We also thank Drs. John McGuire, Richard Moran, and James Coward (Rensselaer Polytechnic Institute) and Dr. Kim Collins (The Johns Hopkins University) for their advice and criticisms.

Registry No. (6*RS*)-H_4PteGlu, 135-16-0; 9-methyl-PteGlu, 2179-16-0; PteGlu, 59-30-3; Pte-L-aspartate, 14798-76-6; Pte-DL-2-aminoadipate, 15677-94-8; Pte-DL-2-aminopimelate, 15565-66-9; Pte-4-aminobutyrate, 15565-66-9; Pte-5-aminovalerate, 105618-34-6; Pte-DL-2-methyl-Glu, 15565-68-1; Pte-kainate, 105618-35-7; Pte-L-2-aminobutyrate, 105618-36-8; Pte-L-2-aminovalerate, 105618-37-9; Pte-DL-homocysteate, 105618-38-0; Pte-L-glutamine, 81672-03-9; Pte-L-ornithine, 105618-39-1; Pte-L-methionine sulfone, 105618-40-4; Pte-L-methionine sulfoxide, 105618-41-5; 4-NH_2-Pte-sulfanilyl-Glu, 64920-89-4; 4-NH_2-H_2Pte-sulfanilyl-Glu, 105618-42-6; $PteGlu_2$, 6807-82-5; 4-NH_2-10-CH_3-$PteGlu_3$, 105618-43-7; 4-NH_2-10-CH_3-$PteGlu_4$, 105618-44-8; Pte-Gly-Glu, 105618-45-9; Pte-β-Asp-Glu, 105618-46-0; Pte-β-Ala-Glu, 105618-47-1; Pte-(4-aminobutyryl)-Glu, 105618-48-2; Pte-(5-aminovaleryl)-Glu, 105618-49-3; PteGlu-[ψ,-CH_2-NH]-Glu, 105618-50-6; (6*RS*)-$H_4PteGlu_2$, 105618-51-7; (6*RS*)-H_4Pte-α-Asp-Glu, 105618-52-8; (6*RS*)-H_4Pte-β-Asp-Glu, 105618-53-9; (6*RS*)-H_4Pte-γ-Glu-Asp, 105618-54-0; (6*RS*)-H_4Pte-Gly-Glu, 105618-55-1; (6*RS*)-H_4Pte-(4-aminobutyryl)-Glu, 83936-03-2; (6*RS*)-5,10-methylene-H_4PteGlu, 6462-99-3; (6*RS*)-10-formyl-H_4PteGlu, 2800-34-2; (6*S*)-H_4PteGlu, 71963-69-4; (6*R*)-5,10-methylene-H_4PteGlu, 31690-11-6; (6*R*)-10-formyl-H_4PteGlu, 74644-66-9; (6*S*)-$H_4PteGlu_2$, 105662-59-7; (6*R*)-5,10-methylene-$H_4PteGlu_2$, 105618-56-2; (6*R*)-10-formyl-$H_4PteGlu_2$, 105662-60-0; (6*S*)-H_4Pte-L-β-Asp-Glu, 105662-61-1; (6*R*)-5,10-methylene-H_4Pte-L-β-Asp-Glu, 105618-57-3; (6*R*)-10-formyl-H_4Pte-L-β-Asp-Glu, 105618-58-4; (6*S*)-H_4Pte-Gly-Glu, 105662-62-2; (6*R*)-5,10-methylene-H_4Pte-Gly-Glu, 105618-59-5; (6*R*)-10-formyl-H_4Pte-Gly-Glu, 105618-60-8; (6*S*)-H_4Pte-β-Ala-Glu, 105618-61-9; (6*R*)-5,10-methylene-H_4Pte-β-Ala-Glu, 105618-62-0; (6*R*)-10-formyl-H_4Pte-β-Ala-Glu, 105618-63-1; (6*S*)-H_4Pte-(4-aminonbutyryl)-Glu, 105662-63-3; (6*R*)-5,10-methylene-H_4Pte-(4-aminobutyryl)-Glu, 105618-64-2; (6*R*)-10-formyl-H_4Pte-(4-aminobutyryl)-Glu, 105618-65-3; (6*S*)-H_4Pte-(5-aminovaleryl)-Glu, 105618-66-4; (6*R*)-5,10-methylene-H_4Pte-(5-aminovaleryl)-Glu, 105618-67-5; (6*R*)-10-formyl-H_4Pte-(5-aminovaleryl)-Glu, 105618-68-6; L-Asp, 56-84-8; H_2PteGlu, 4033-27-6; $H_2PteGlu_2$, 15582-21-5; H_2Pte-β-Ala-Glu, 105618-69-7; H_2Pte-(4-aminobutyryl)-Glu, 105618-70-0; H_2Pte-(5-aminovaleryl)-Glu, 105618-71-1; 9-methyl-H_2PteGlu, 17863-76-2; (6*S*)-9-methyl-H_4PteGlu, 17863-76-2;

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