First Use of the Taylor Pteridine Synthesis as a Route to Polyglutamate Derivatives of Antifolates[1]

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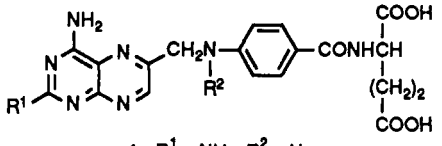
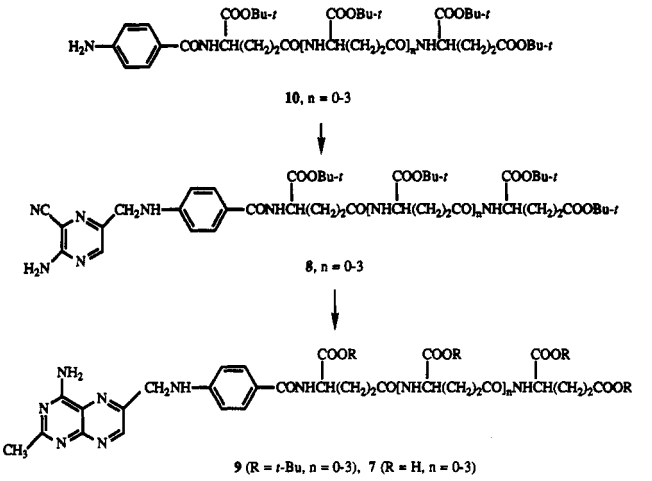
The di- through penta-γ-L-glutamates of 2-desamino-2-methylaminopterin, a new antifolate with a novel mechanism of action requiring γ-polyglutamylation for biological activity, were prepared. α-*tert*-Butyl γ-methyl L-glutamate was condensed with 4-nitrobenzoyl chloride, the methyl ester selectively hydrolyzed with base, and the product condensed with di-*tert*-butyl L-glutamate to obtain tri-*tert*-butyl *N*-(4-nitrobenzoyl)-γ-L-glutamyl-L-glutamate. Reduction of the nitro group followed by reaction with 2-amino-5-(chloromethyl)-pyrazine-3-carbonitrile yielded tri-*tert*-butyl [4-[[(2-amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-L-γ-glutamyl-L-glutamate, which was heated with acetamidine acetate to form tri-*tert*-butyl *N*-[4-[[(4-amino-2-methylpteridine-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-L-glutamate. Removal of the ester groups with trifluoroacetic acid then gave 2-desamino-2-methylaminopterin diglutamate. A similar sequence was employed to convert esterified oligomers with three, four, and five glutamyl residues to 2-desamino-2-methylaminopterin tri-, tetra-, and pentaglutamate. This is the first example of the preparation of the polyglutamates of an antifolate via the Taylor pteridine synthesis.

Analogues of the classical folate antagonists aminopterin (**1**) and methotrexate (**2**) with hydrogen (**3**, **5**) or methyl (**4**, **6**) in place of the 2-amino group were recently reported[2,3] to inhibit tumor cell growth in culture despite the fact that they were weak inhibitors of isolated dihydrofolate reductase, the primary target enzyme for 2,4-diamino antifolates.[4] The desamino analogues were found to be substrates for the enzyme folylpolyglutamate synthetase, which is responsible for the intracellular conversion of classical antifolates, as well as natural folate cofactors, to γ-polyglutamates.[5] These conjugates are considered to play a critical role in the biological activity of methotrexate because (a) they do not efflux from cells and (b) they inhibit two other important enzymes of one-carbon metabolism, namely thymidylate synthase[6a] and glycinamide ribotide transformylase.[6b] Since the growth-inhibitory effect of the desaminoaminopterin and desaminomethotrexate monoglutamates could not be explained on the basis of their interaction with dihydrofolate reductase, which was very weak, we postulated that intracellularly formed γ-polyglutamates are the species responsible for biological activity.[2]



1: $R^1 = NH_2$, $R^2 = H$
2: $R^1 = NH_2$, $R^2 = Me$
3: $R^1 = R^2 = H$
4: $R^1 = Me$, $R^2 = H$
5: $R^1 = H$, $R^2 = Me$
6: $R^1 = R^2 = Me$

Scheme I



9 (R = *t*-Bu, n = 0-3), **7** (R = H, n = 0-3)

To determine which of the folate pathway enzymes might be most sensitive to desaminoaminopterin and desaminomethotrexate polyglutamates it was necessary to develop a chemical route to these compounds. The present paper reports the synthesis of the di- through pentaglutamates of **4** by an extension of the method we used earlier to prepare the monoglutamate.[2,3] The general structure of these polyglutamates, **7** (n = 0–3), as well as our overall synthetic plan, are shown in Scheme I. Key steps were the annulation of a series of *tert*-butyl *N*-[4-[[(2-amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-oligo-γ-L-glutamates (**8**, n = 0–3) with acetamidine and the deprotection of the cyclized products **9** (n = 0–3) with acid. The 2-amino-3-cyanopyrazine intermediates **8** were prepared by alkylation of *tert*-butyl *N*-(4-aminobenzoyl)-oligo-γ-L-glutamates (**10**, n = 0–3) with 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile. It should be noted that, in our synthesis, pteridine ring closure is the penultimate step, whereas other literature routes typically involve linkage of the oligoglutamate side chain, stepwise or in one piece, to a preformed pteridine or deazapteridine moiety.[7-14]

(1) Paper 45 in this series; for previous paper, see: Rosowsky, A.; Forsch, R. A.; Bader, H.; Freisheim, J. H. *J. Med. Chem.*, in press.
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(3) Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Freisheim, J. H. *J. Med. Chem.* **1991**, *34*, 227.
(4) For a discussion of current views concerning the direct and indirect effects of dihydrofolate reductase inhibition on cellular one-carbon metabolism, see: (a) Seither, R. L.; Trent, D. F.; Mikulecky, D. C.; Rape, T. J.; Goldman, I. D. *J. Biol. Chem.* **1989**, *264*, 17016.
(5) For leading references on the important role of γ-polyglutamylation in the biological action of antifolates, see: Johnson, T. B.; Nair, M. G.; Galivan, J. *Cancer Res.* **1988**, *48*, 2426.
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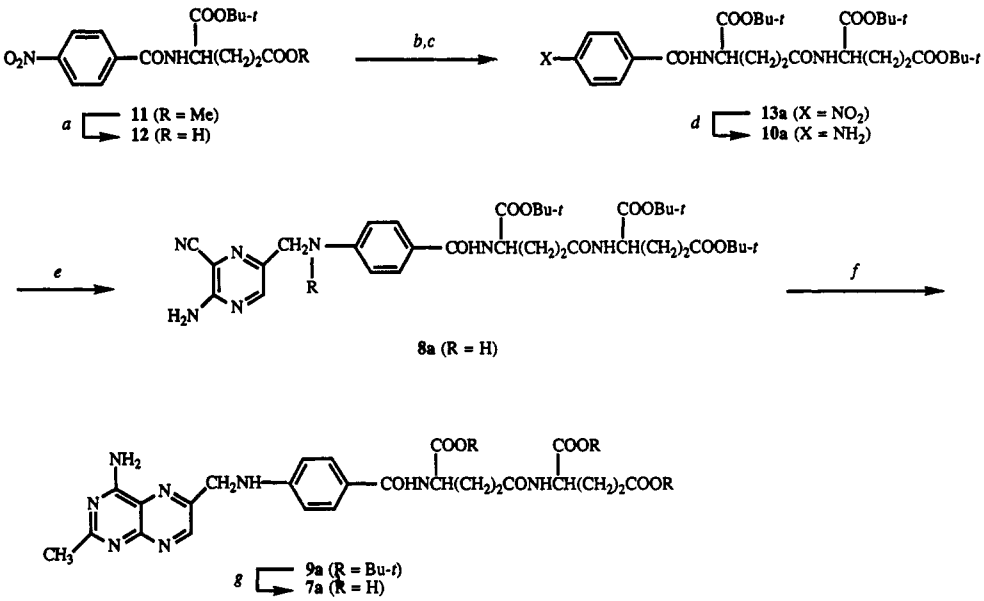
Scheme II[a]



[a] Key: a, NaOH; b, *i*-BuOCOCl/*N*-methylmorpholine; c, $H_2NCH(COO\text{-}t\text{-}Bu)(CH_2)_2COO\text{-}t\text{-}Bu$; d, H_2/PtO_2; e, 2-amino-5-(chloromethyl)-pyrazine-3-carbonitrile/*i*-Pr_2EtN; f, $CH_3C(=NH)NH_2 \cdot AcOH$; g, 1:2 CF_3CO_2H/CH_2Cl_2.

The synthesis of the diglutamate **7a** served as the model for the rest of the work and is detailed in Scheme II. α-*tert*-Butyl γ-methyl L-glutamate[15] was condensed with 4-nitrobenzoyl chloride, and the methyl ester group was selectively removed with base to obtain the previously unknown compounds α-*tert*-butyl γ-methyl *N*-(4-nitrobenzoyl)-L-glutamate (**11**, 93%) and α-*tert*-butyl *N*-(4-nitrobenzoyl)-L-glutamate (**12**, 71%), respectively. Coupling of **12** with di-*tert*-butyl L-glutamate in the presence of diphenyl phosphorazidate followed by catalytic reduction of the nitro group then gave the diglutamates **13a** (100%) and **10a** (84%). Monoalkylation of **10a** with an equimolar amount of 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile[16] and diisopropylethylamine in *N*-methylpyrrolidin-2-one at room temperature for 7 days gave the pyrazine **8a** (39%). Ring closure of **8a** with acetamidine acetate[17] in 2-methoxyethanol (1.75 h at reflux temperature)[2,3] gave the esterified pteridine **9a** (47%). This was an improvement over 2-ethoxyethanol as the solvent,[2] which gave considerable discoloration and a lower yield of product (24%). The ester groups were successfully removed by acidolysis with trifluoroacetic acid in methylene chloride (1:2 mixture, 3 h at room temperature).[2,3] Preparative HPLC (C_{18} silica gel, 25% MeCN in 0.1 M ammonium acetate, pH 6.5) and reprecipitation of the principal fraction from ammonia with acetic acid afforded analytically pure **7a** (31%) as the hydrated free acid. The product showed UV absorption maxima (λ_{max} (0.1 N NaOH) 221, 246, 292 nm; λ_{max} (0.1 N HCl) 228, 294 nm) similar to those previously reported for the monoglutamate **4**,[3] as well as a mass spectral peak in agreement with the theoretical M + 1 value.

It is important to note that the progress of cleavage of **7a** and the other polyglutamate esters with trifluoroacetic acid had to be carefully monitored by HPLC to maximize product formation. Several transient peaks other than that of the product were seen as the reaction progressed, some of which appeared to represent incomplete hydrolysis. Some evidence of product decomposition was also noted. Thus, a reaction time had to be empirically established for each ester that was optimal for that particular compound (see Experimental Section).

The synthesis of the tri- through pentaglutamates **7b–d** (Scheme III) was similar to that of **7a** except for the preparation of the *N*-(4-aminobenzoyl)oligo-γ-L-glutamates **10b–d** and the *N*-(4-nitrobenzoyl)oligo-γ-L-glutamates **13b–d**, which involved acylation of preformed tri-, tetra-, and pentaglutamate esters (**15b–d**) with 4-nitrobenzoyl chloride followed by catalytic reduction of the nitro group. The known oligoglutamate *tert*-butyl esters[8a,9] were prepared by iterative mixed carboxylic–carbonic anhydride coupling. For example, di-*tert*-butyl L-glutamate was condensed with α-*tert*-butyl *N*-(benzyloxycarbonyl)-L-glutamate with the aid of isobutyl chloroformate and *N*-methylmorpholine, the *N*-benzyloxycarbonyl group was removed by catalytic hydrogenation (10% Pd–C) to obtain tri-*tert*-butyl γ-L-glutamyl-L-glutamate (**15a**), and a second cycle of mixed anhydride coupling and catalytic hydrogenation was performed to obtain tetra-*tert*-butyl γ-L-glutamyl-γ-L-glutamyl-L-glutamate (**15b**) as an oil. The corresponding tetraglutamate (**15c**) and pentaglutamate (**15d**) were prepared in the same manner and were obtained as crystalline solids. Compounds **15a–d** have been cited briefly before in the literature,[8a,9] but microanalytical data were not presented. The *N*-(4-nitrobenzoyl) derivatives **13b–d** and *N*-(4-aminobenzoyl) derivatives **10b–d** have not been described until now.

Treatment of 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile with a stoichiometric amount of **10b** and diisopropylethylamine in *N*-methylpyrrolidin-2-one at room

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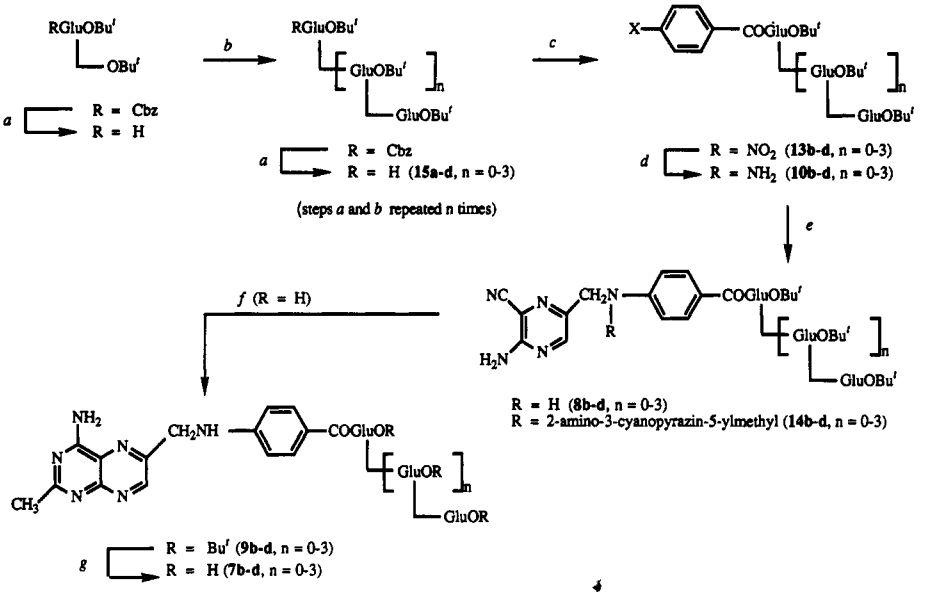
Scheme III[a]



[a] Key: a, H_2/Pd–C; b, CbzGluO-*t*-Bu/$(PhO)_2P(=O)N_3$; c, 4-$NO_2C_6H_4COCl$/*N*-methylmorpholine; d, H_2/Pd–C; e, 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile; f, $CH_3C(=NH)NH_2$·AcOH; g, CF_3CO_2H/CH_2Cl_2.

temperature for ca. 4 days afforded a mixture of the monoalkylated product **8b** (54% yield) and a more polar byproduct that was separated from **8b** by column chromatography on silica gel and identified as **14b** (19% yield). The structure of **14b** was evident from its microanalytical data, its IR spectrum, in which the CN peak at 2220 cm^{-1} was much stronger than the corresponding peak for **8b**, and its ^1H NMR spectrum, which showed the pyrazine C_6 protons (δ 8.08) and the 3′- and 5′-protons (δ 6.65) and 2′- and 6′-protons (δ 7.65) of the phenyl ring in a ratio of 1:1:1 rather than 1:2:2 (as in **8b**). A similar alkylation reaction with **10c** and **10d** afforded the monoalkylated products **8c** (42%) and **8d** (50%) along with the dialkylated products **14c** (21%) and **14d** (20%). Approximately 20% of the starting amine was also recovered in each instance. It is interesting to note that mixtures of mono- and dialkylated products were formed even though a stoichiometric amount of 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile was used and was added to the amine in small portions. The formation of the dialkylated product indicates that the monoalkylated compound is more reactive than the starting material. Dialkylation of primary amines by 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile, which was not mentioned in earlier papers describing the chemistry of this compound,[16a,b] can only occur in the synthsis of N^{10}-unsubstituted analogues and is obviously not a problem in the synthesis of N^{10}-alkyl derivatives (e.g., methotrexate polyglutamate analogues). Although protection of the NH_2 group in **10b–d** was considered as a means of avoiding the dialkylation problem, the extra steps involved in protection and deprotection discouraged us from pursuing this approach.

Ring closure of the amino nitriles **8b–d** with excess acetamidine acetate[17] in refluxing 2-methoxyethanol proceeded in roughly 60% yield. This yield was somewhat higher than that obtained from **8a** and probably reflects the fact that acetamidine acetate was used in larger excess and was added in several portions (see Experimental Section). Heating acetamidine at the boiling temperature of 2-methoxyethanol (125 °C) is likely to result in some self-condensation, thereby rendering it unavailable for the annulation reaction. It was previously established that annulation of the monoglutamate **8a** by heating with formadidine or acetamidine acetate proceeds without racemization of the amino acid side chain,[2] in contrast to the use of guanidine, which is known to be accompanied by extensive racemization of the glutamate moiety.[18] Since it is unlikely that the oligoglutamates **8b–d** behave differently from the monoglutamate **8a** under the identical ring closure conditions, it is reasonable to assume that the glutamic acid residues in **9b–d** have retained their original L-configuration.

Cleavage of the ester groups in **9b–d** with 1:2 mixtures of trifluoroacetic acid and methylene chloride[19] proceeded uneventfully, affording **7b** (45%), **7c** (36%), and **7d** (49%). The triglutamate **7b** was purified by preparative HPLC without subsequent precipitation from ammonia with acetic acid and was therefore obtained as a hydrated monoammonium salt after freeze-drying, rather than as the free acid. The tetraglutamate **7c** was purified by preparative HPLC followed by precipitation from ammonia with acetic acid; the pentaglutamate **7d** was purified similarly except for an additional gel filtration step (see Experimental Section). In this instance, the products were obtained as hydrated free acids. The structures of **7b–d** were corroborated by microchemical analysis. In addition, the mass spectra (negative FAB mode) of **7c** and **7d** contained major peaks at the expected M – 1 values of 826 and 955, respectively. That these compounds were all 2-desamino-2-methylpteridines was also clear from their UV spectra, which were virtually identical with those of the monoglutamate **4** and diglutamate **7a**.

In summary, a method of preparation of the di- through pentaglutamates of 2-desamino-2-methylaminopterin has been developed that differs from those in the literature[7–14] in that closure of the pteridine ring occurs at the penultimate step, making it possible to synthesize a variety of analogues modified at C_2 of the pteridine moiety from amidines and polyglutamylated amino nitrile precursors. The availability of the polyglutamates described in this

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(19) Although alternative acidolysis conditions for the cleavage of the *tert*-butyl ester groups in **9b–d** were not examined, it is worth noting that aqueous HCl has recently been reported[13c] to deprotect other *tert*-butyl oligoglutamate derivatives in good yield.

paper will enable them to be tested as inhibitors of various enzymes of one-carbon metabolism. It is expected that these studies will lead to identification of the biochemical locus of action of 2-desamino-2-methylaminopterin and a better understanding of its novel mode of activity.

Experimental Section

Where microanalyses indicate the presence of an organic solvent such as Et_2O or CH_2Cl_2, appropriate ^1H NMR signals were observed. TLC analyses were on fluorescent Baker Si250F silica gel plates, Eastman 13181 silica gel sheets, or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV illumination or with the aid of ninhydrin. Column chromatography was on Baker 3405 (60-200 mesh), Baker 7024-1 Flash silica gel (40-μm particle size), or Woelm neutral alumina. Analytical HPLC was on a Waters C_{18} radial compression cartridge column (5-μm particle size, 0.5 mm × 10 cm) and preparative HPLC on a Dynamax Macro C_{18} column (21.4 mm × 25 cm). Melting points were taken on a hot-stage apparatus or in Pyrex capillary tubes and are not corrected. α-*tert*-Butyl γ-methyl-L-glutamate[15] and *N*-(benzyloxycarbonyl)oligo-γ-L-glutamic acid tetra-, penta-, or hexa-*tert*-butyl esters[8a] were prepared according to the literature. Di-*tert*-butyl *N*-(benzyloxycarbonyl)-L-glutamate and α-*tert*-butyl *N*-(benzyloxycarbonyl)-L-glutamate were obtained from Bachem (Torrance, CA); other chemicals were from Aldrich, Milwaukee, WI. Solvents for moisture-sensitive reactions were dried over Linde 4A molecular sieves (Fisher, Boston, MA). Unless specified otherwise, organic phases after extraction were dried over $MgSO_4$. Microchemical analyses were performed by Robertson Laboratory, Madison, NJ.

Synthesis of Oligo-γ-L-glutamic Acids. A solution of the appropriate *N*-(benzyloxycarbonyl)oligo-γ-L-glutamic acid tetra-, penta-, or hexa-*tert*-butyl ester (1 mmol) in a mixture of MeOH (25 mL) and glacial AcOH (8 mL) was shaken with H_2 and 10% Pd–C (0.15 g) in a Parr low-pressure apparatus (initial pressure 50 lb/in.^{2}) for 1.5 h. The catalyst was filtered, and the solution was evaporated to dryness. The residue was redissolved in $CHCl_3$, and the solution was washed with H_2O and stirred with 1 N NaOH until the pH of the aqueous phase was alkaline (this step was omitted for the triglutamate, resulting in isolation of this compound as the acetate salt). The organic phase was then rinsed to neutrality with H_2O, dried ($MgSO_4$), and evaporated. The residue was dried to constant weight on a rotary evaporator at 50 °C (bath temperature) if the product was an oil (n = 2, 3) or was recrystallized from mixtures of Et_2O and hexanes and dried in vacuo at 25 °C if it was a solid (n = 4, 5): IR (thin film or KBr) 3310, 1730 (ester C=O), 1645 (amide C=O) cm^{-1}. Specific compounds synthesized by this procedure are listed in the following text.

Tri-*tert*-butyl γ-L-glutamyl-L-glutamate (15a): 76%; oil; TLC R_f 0.67 (silica gel, 9:1 $CHCl_3$–MeOH). Anal. Calcd for $C_{22}H_{40}N_2O_7$: C, 59.43; H, 9.07; N, 6.30. Found: C, 59.36; H, 8.83; N, 5.97.

Tetra-*tert*-butyl γ-L-glutamyl-γ-L-glutamyl-L-glutamate (15b): 98%; oil; TLC R_f 0.37 (silica gel, 9:1 $CHCl_3$–MeOH). Anal. Calcd for $C_{31}H_{55}N_3O_{10}\cdot CH_3CO_2H\cdot H_2O$: C, 55.99; H, 8.69; N, 5.93. Found: C, 55.79; H, 8.55; N, 6.13.

Penta-*tert*-butyl γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (15c): 88%; mp 119–119.5 °C; TLC R_f 0.43 (silica gel, 9:1 $CHCl_3$–MeOH). Anal. Calcd for $C_{40}H_{70}N_4O_{13}$: C, 58.94; H, 8.66; N, 6.87. Found: C, 59.06; H, 8.80; N, 6.68.

Hexa-*tert*-butyl γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (15d): 98%; mp 133–135 °C; TLC R_f 0.41 (silica gel, 9:1 $CHCl_3$–MeOH). Anal. Calcd for $C_{49}H_{85}N_5O_{16}$: C, 58.84; H, 8.57; N, 7.00. Found: C, 58.60; N, 8.49; N, 6.82.

Synthesis of *N*-(4-Nitrobenzoyl)-L-glutamic Acid Esters. A. α-*tert*-Butyl γ-Methyl *N*-(4-Nitrobenzoyl)-L-glutamate (11). To a solution of α-*tert*-butyl γ-methyl L-glutamate (1.09 g, 0.005 mol) and 4-nitrobenzoyl chloride (0.93 g, 0.005 mol) in CH_2Cl_2 (50 mL) cooled to 0 °C was slowly added Et_3N (1.51 g, 0.015 mol). After being kept at room temperature for 18 h, the reaction mixture was cooled to −20 °C and washed rapidly with H_2O, followed by 0.1 N HCl (2 × 35 mL), H_2O, and saturated $NaHCO_3$. The organic layer was dried and evaporated to a thick oil (1.7 g, 93%): TLC R_f 0.15 (silica gel, $CHCl_3$); IR (thin film) 3340, 1745, 1735 (ester C=O), 1670 (amide C=O), 1605 (aromatic), 1535 and 1355 (NO_2) cm^{-1}; NMR ($CDCl_3$) δ 1.55 (s, 9 H, *t*-Bu), 2.0–2.5 (m, 4 H, β- and γ-CH_2), 3.66 (s, 3 H, OMe), 4.65 (m, 1 H, α-CH), 5.25 (s, 1 H, NH), 7.93 (d, J = 4.5 Hz, 2 H, aromatic), 8.26 (d, J = 4.5 Hz, 2 H, aromatic). Anal. Calcd for $C_{17}H_{22}N_2O_7$: C, 55.72; H, 6.05; N, 7.65. Found: C, 55.57; H, 6.16; N, 7.48.

B. α-*tert*-Butyl *N*-(4-Nitrobenzoyl)-L-glutamate (12). A solution of 11 (1.67 g, 4.56 mmol) in a mixture of MeOH (40 mL) and 1 N NaOH (10 mL) was stirred at room temperature for 1.5 h. The MeOH was evaporated under reduced pressure, H_2O was added, and the pH was adjusted to 4.5 with 10% AcOH. The precipitated gum was redissolved in $CHCl_3$, the solution was evaporated to dryness, and the residue was dried under vacuum to constant weight: yield 1.15 g (71%); TLC R_f 0.52 (silica gel, 5:1 $CHCl_3$–MeOH); IR (KBr) 3450, 1730 (ester C=O), 1690 (acid C=O), 1660 (amide C=O), 1600 aromatic, 1535 and 1350 (NO_2) cm^{-1} (the spectrum was obtained from crystals that formed spontaneously during washing of the gummy product with CS_2); NMR ($CDCl_3$) δ 1.48 (s, 9 H, *t*-Bu), 2.0–3.0 (m, 4 H, β- and γ-CH_2), 4.65 (m, 1 H, α-CH), 7.86 (d, J = 4 Hz, 2 H, aromatic), 8.20 (d, J = 4 Hz, 2 H, aromatic). The analytical sample was dried over P_2O_5 at 60 °C overnight. Anal. Calcd for $C_{16}H_{20}N_2O_7$: C, 54.54; H, 5.72; N, 7.95. Found: C, 54.15; H, 5.63; N, 7.63.

C. Tri-*tert*-butyl *N*-(4-Nitrobenzoyl)-γ-L-glutamyl-L-glutamate (13a). A solution of α-*tert*-butyl *N*-(benzyloxycarbonyl)-L-glutamate (1.12 g, 3.18 mmol) and di-*tert*-butyl L-glutamate hydrochloride (0.941 g, 3.18 mmol) was cooled to 0 °C, and diphenyl phosphorazidate (0.963 g, 3.5 mmol) followed by Et_3N (1.06 g, 10.5 mmol) were added dropwise. After being kept at 0 °C for 18 h and then at room temperature for 72 h, the reaction mixture was poured onto ice (200 mL) and the precipitated gum was extracted with $CHCl_3$. The $CHCl_3$ extract was evaporated to dryness under reduced pressure, and the residue was redissolved in a small volume of $CHCl_3$ and applied onto a silica gel column (75 g, 3.0 × 26 cm) that was eluted with $CHCl_3$. Fractions showing a TLC spot with R_f 0.68 (silica gel, 95:5 $CHCl_3$–MeOH) were pooled and evaporated, and the residue was dried to constant weight at 60 °C under reduced pressure to obtain an oil (1.92 g, 100%): NMR ($CDCl_3$) δ 1.41 (s, 18 H, α- and γ-*t*-Bu), 1.48 (s, 9 H, α-*t*-Bu), 1.75–2.6 (m, 8 H, β- and γ-CH_2), 4.46 (m, 2 H, α-CH), 6.5 (m, 1 H, NH), 8.0 (d, J = 4 Hz, 2 H, aromatic), 8.3 (d, J = 4 Hz, 2 H, aromatic). Anal. Calcd for $C_{29}H_{43}N_3O_{10}\cdot 0.5H_2O$: C, 57.70; H, 7.35; N, 6.97. Found: C, 57.95; H, 7.29; N, 6.95.

D. Penta-*tert*-butyl *N*-(4-Nitrobenzoyl)-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (13d). *N*-Methylmorpholine (0.303 g, 3.0 mmol) was added to a solution of **15c** (0.76 g, 0.937 mmol) and 4-nitrobenzoyl chloride (0.186 g, 1.0 mmol) in CH_2Cl_2 (35 mL) at 0 °C. After being kept at 25 °C for 1.5 h, the solution was washed with 0.1 N HCl, neutralized with saturated $NaHCO_3$, dried, and evaporated. The residue was redissolved in 1:1 EtOAc–$CHCl_3$ and the solution applied onto a column of silica gel (40 g, 2.0 × 28 cm) that was eluted with the same mixture. Fractions showing a TLC spot with R_f 0.24 (silica gel, 1:1 EtOAc–$CHCl_3$) were pooled and evaporated to constant weight at 70 °C (bath temperature) on a rotary evaporator to obtain a colorless solid (811 mg, 90%): mp 66–68 °C; IR (KBr) 3380, 2980, 2940, 1740 (ester C=O), 1660 (amide C=O), 1530 and 1370 (NO_2) cm^{-1}. Anal. Calcd for $C_{47}H_{73}N_5O_{16}$: C, 58.55; N, 7.63; N, 7.26. Found: C, 58.36; H, 7.57; N, 7.07.

E. Tetra-*tert*-butyl *N*-(4-nitrobenzoyl)-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (13b): 93%; mp 52–56 °C; TLC R_f 0.56 (silica gel, 1:1 EtOAc–$CHCl_3$). Anal. Calcd for $C_{38}H_{58}N_4O_{13}$: C, 58.59; H, 7.51; N, 7.19. Found: C, 58.98; H, 7.76; N, 6.95.

F. Hexa-*tert*-butyl *N*-(4-nitrobenzoyl)-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (13e): 97% (chromatography omitted); mp 70–72 °C; TLC R_f 0.21 (silica gel, 1:1 EtOAc–$CHCl_3$). Anal. Calcd for $C_{56}H_{88}N_6O_{19}\cdot 0.6H_2O$: C, 57.97; H, 7.75; N, 7.24. Found: C, 57.96; H, 7.69; N, 7.04.

Synthesis of *N*-(4-Aminobenzoyl)-L-glutamic Acid Esters. A. Tri-*tert*-butyl *N*-(4-Aminobenzoyl)-γ-L-glutamyl-L-glutamate (10a). A solution of **13a** (1.93 g, 3.2 mmol) in a mixture of EtOH (15 mL) and glacial AcOH (5 mL) was shaken with H_2 and PtO_2 (20 mg) in a Parr low-pressure apparatus (initial pressure 48 lbs/in^2) for 2 h. After removal of the catalyst, the solution

(TLC R_f 0.39, silica gel, 95:5 $CHCl_3$–MeOH) was evaporated to dryness and the residue was redissolved in $CHCl_3$ and applied onto a silica gel column (75 g, 3.0 × 25 cm) that was prepared and eluted with 20:20:1 $CHCl_3$–MeCN–MeOH. Fractions showing a TLC spot with R_f 0.85 (silica gel, 20:20:1 $CHCl_3$–MeCN–MeOH) were combined and evaporated. The solid residue (1.52 g, 84%) was redissolved in a small volume of CH_2Cl_2, and excess Et_2O was added to obtain a colorless solid (0.77 g) after drying at 60 °C over P_2O_5: mp 128–129 °C; IR (KBr) 3390, 1735 (ester C=O), 1650 (amide C=O), 1615 (aromatic) cm^{-1}. Evaporation of the mother liquor afforded an equally pure second crop (0.72 g). Anal. Calcd for $C_{29}H_{45}N_3O_8$: C, 61.79; H, 8.05; N, 7.45. Found: C, 61.87; H, 7.76; N, 7.43.

B. Tetra-*tert*-butyl *N*-(4-aminobenzoyl)-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (10b): 52%; mp 62–65 °C; TLC R_f 0.22 (silica gel, 1:1 EtOAc–$CHCl_3$). Anal. Calcd for $C_{38}H_{60}N_4O_{11}\cdot Et_2O$: C, 61.29; H, 8.57; N, 6.80. Found: C, 61.15; H, 8.18; N, 6.69.

C. Penta-*tert*-butyl *N*-(4-aminobenzoyl)-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (10c): 95%; mp 77–78 °C; TLC R_f 0.13 (silica gel, 1:1 EtOAc–$CHCl_3$). Anal. Calcd for $C_{47}H_{75}N_5O_{14}$: C, 60.43; H, 8.09; N, 7.50. Found: 60.29; H, 7.94; N, 7.06.

D. Hexa-*tert*-butyl *N*-(4-aminobenzoyl)-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (10d): 96%; mp 83–86 °C; TLC R_f 0.07 (silica gel, 1:1 EtOAc–$CHCl_3$). Anal. Calcd for $C_{56}H_{90}N_6O_{17}\cdot 0.75H_2O$: C, 59.36; H, 8.07; N, 7.42. Found: C, 59.56; H, 8.02; N, 7.25.

Alkylations with 2-Amino-5-(chloromethyl)pyrazine-3-carbonitrile. A. Tri-*tert*-butyl [4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-L-γ-glutamyl-L-glutamate (8a). 2-Amino-5-(chloromethyl)pyrazine-3-carbonitrile (202 mg, 1.2 mmol) was added in several portions over a period of 15 min to a stirred solution of **10a** (675 mg, 1.2 mmol) and diisopropylethylamine (186 mg, 1.44 mmol) in *N*-methylpyrrolidin-2-one (30 mL). After 7 days at room temperature, the solvent was removed in vacuo. The residue was redissolved in $CHCl_3$ (70 mL), and the solution was washed with H_2O (2 × 40 mL), dried, and evaporated. The crude product was taken up in Et_2O and applied onto a column of neutral alumina (20 g, 1.5 × 14 cm) that was eluted sequentially with Et_2O, 3:1 and 1:1 Et_2O–acetone mixtures, and finally acetone. Fractions showing a TLC spot with R_f 0.34 (silica gel, 95:5 $CHCl_3$–MeOH) were pooled, evaporated, and applied onto a silica gel column (40-μm particle size, 15 g, 1.5 × 23 cm) in $CHCl_3$ solution. The column was eluted with 99:1 and 97:3 $CHCl_3$–MeOH, and TLC homogeneous fractions were pooled and evaporated to obtain a solid (0.327 g, 39%): mp 152–153 °C; IR (KBr) 3395, 3220, 2980, 2220 (CN), 1760 (ester C=O), 1640 (amide C=O), 1610 (aromatic), 1520, 1390, 1370, 1255, 1177, 840, 760 cm^{-1}; NMR ($CDCl_3$) δ 1.38 (s, 18 H, γ-*t*-Bu and α-*t*-Bu), 1.41 (s, 9 H, α-*t*-Bu), 1.7–2.4 (m, 8 H, β- and γ-CH_2), 4.38 (m, 4 H, NH_2 and α-CH), 5.36 (m, 2 H, NCH_2), 6.50 (d, J = 9 Hz, 2 H, aromatic), 7.65 (d, J = 9 Hz, 2 H, aromatic), 6.5–7.5 (m, 2 H, amide NH), 8.20 (s, 1 H, C_6-H). The analytical sample was precipitated from a mixture of CH_2Cl_2 and acetone. Anal. Calcd for $C_{35}H_{49}N_7O_8$: C, 60.41; H, 7.10; N, 14.09. Found: C, 60.11; H, 7.08; N, 13.80.

B. Tetra-*tert*-butyl [4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (8b) and Tetra-*tert*-butyl [4-[Bis[(2-amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (14b). 2-Amino-5-(chloromethyl)-pyrazine-3-carbonitrile (86 mg, 0.507 mmol) was added in small portions over 5 min to a stirred solution of **10b** (380 mg, 0.462 mmol) and diisopropylethylamine (79 mg, 0.507 mmol) in MeCN (20 mL). After another 10 min of stirring, the solution was left at room temperature for 42 h. Analysis by TLC (silica gel, EtOAc) revealed three spots with R_f values of 0.68 (unchanged **10b**), 0.55 (found to be **8b**), and 0.44 (found to be **14b**). The solvent was evaporated and the residue redissolved in $CHCl_3$. The solution was washed twice with H_2O and adsorbed onto a silica gel column (40-μm particle size, 40 g, 2 × 26 cm) that was eluted with EtOAc. Fractions showing only the TLC spot with R_f 0.68 were pooled and evaporated. Other fractions still showing three spots were pooled and chromatographed twice more on silica gel, using $CHCl_3$ and 99:1 $CHCl_3$–MeOH as the eluents. Appropriate TLC homogeneous fractions from the second and third columns were pooled and evaporated, and the residues were collected and dried to constant weight over P_2O_5 at 25 °C. In the case of the pooled fractions with R_f 0.44, mixtures of Et_2O and CH_2Cl_2 were used to facilitate collection and transfer to the solid prior to drying. The following products were obtained. (i) **10b**: 72 mg, 19% recovery; TLC R_f 0.68 (silica gel, EtOAc). (ii) **8b**: yellow powder (224 mg, 54%); mp 75–78 °C; R_f 0.55; IR (KBr) 3380, 2980, 2930, 2220 (CN), 1725 (ester C=O), 1635 (amide C=O), 1605 (aromatic) cm^{-1}; NMR ($CDCl_3$) δ 1.43, 1.45, and 1.48 (singlets, 36 H, *t*-Bu), 1.8–2.7 (m, 12 H, β- and γ-CH_2), 4.37 (m, 6 H, CH_2N and NH), 5.47 (m, 3 H, α-CH), 6.60 (d, J = 9 Hz, $C_{3'}$- and C_5-H), 6.95 (m, 2 H, NH_2), 7.7 (d, J = 9 Hz, 2 H, $C_{2'}$- and C_6-H), 8.21 (C_6-H). Anal. Calcd for $C_{44}H_{64}N_8O_{11}\cdot 0.75H_2O$: C, 59.07; H, 7.38; N, 12.53. Found: C, 59.24; H, 7.26, N, 12.14. (iii) **14b**: yellow powder (99 mg, 19%); mp 78–81 °C; TLC R_f 0.44 (silica gel, EtOAc); IR (KBr) 3340, 3220, 2980, 2940, 2220 (CN, very strong), 1730 (ester C=O), 1635 (amide C=O), 1610 (aromatic) cm^{-1}; NMR ($CDCl_3$) δ 1.40 (s, 36 H, *t*-Bu), 1.6–2.5 (m, 12 H, β- and γ-CH_2), 4.35 (m, 4 H, NH), 4.67 (s, 4 H, CH_2N), 5.48 (m, 3 H, α-CH), 6.65 (d, J = 9 Hz, 2 H, $C_{3'}$- and C_5-H), 6.75–7.2 (m, 4 H, NH_2), 7.65 (d, J = 9 Hz, $C_{2'}$- and C_6-H), 8.08 (s, 2 H, C_6-H). Anal. Calcd for $C_{50}H_{68}N_{12}O_{11}\cdot 0.75Et_2O\cdot 0.75CH_2Cl_2$) C, 57.00; H, 6.85; N, 14.84. Found: C, 56.80; H, 6.77; N, 14.74.

C. Penta-*tert*-butyl [4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (8c) and Penta-*tert*-butyl [4-[Bis[(2-amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (14c). After silica gel chromatography as described previously for **8b**, an additional purification step involving flash chromatography on silica gel with 99:1 $CHCl_3$–MeOH as the eluent gave the following products. (i) recovered **10c**: 27%; (ii) **8c**: yellow powder (42% yield); mp 78–80 °C; TLC R_f 0.41 (silica gel, EtOAc). Anal. Calcd for $C_{53}H_{79}N_9O_{14}\cdot 0.5MeOH$: C, 59.37; H, 7.54; N, 11.65. Found: C, 59.55; H, 7.45; N, 11.20. (iii) **14c**: yellow powder (21% yield); mp 93–97 °C; TLC R_f 0.34 (silica gel, EtOAc). Anal. Calcd for $C_{59}H_{83}N_{13}O_{14}\cdot H_2O$: C, 58.25; H, 7.04; N, 14.97. Found: C, 58.36; H, 7.13; N, 14.55.

D. Hexa-*tert*-butyl [4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (8d) and Hexa-*tert*-butyl [4-[Bis[(2-amino-3-cyanopyrazin-5-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (14d). Separation on neutral alumina with $CHCl_3$ followed by 99:1 and 95:5 $CHCl_3$–MeOH as eluents gave the following products. (i) recovered **10d**: 24%. (ii) **8d**: yellow powder (50% yield); mp 88–90 °C; TLC R_f 0.60 (silica gel, 1:1 MeCN–$CHCl_3$). Anal. Calcd for $C_{62}H_{94}N_{10}O_{17}\cdot H_2O$: C, 58.65; H, 7.62; N, 11.03. Found: C, 58.75; H, 7.52; N, 10.99. (iii) **14d**: yellow powder (20% yield); mp 92–94.5 °C; TLC R_f 0.46 (silica 1:1 MeCN–$CHCl_3$). Anal. Calcd for $C_{68}H_{98}N_{14}O_{17}\cdot H_2O$: C, 58.27; H, 7.19; N, 13.99. Found: C, 58.44; N, 7.23; N, 13.81.

Ring Closure Reactions with Acetamidine Acetate. A. Tri-*tert*-butyl [4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-L-glutamate (9a). A mixture of **8a** (327 mg, 0.47 mmol), acetamidine acetate (278 mg, 2.35 mmol), and 2-methoxyethanol (10 mL) was heated under reflux for 1.75 h. The solvent was distilled under reduced pressure, and the residue was partitioned between $CHCl_3$ and H_2O (50 mL each). The organic layer was dried, concentrated to a small volume, and applied onto a silica gel column (40-μm particle size, 7 g, 1 × 20 cm). The column was eluted first with $CHCl_3$ and then with 97:3 and 95:5 $CHCl_3$–MeOH. Fractions showing a TLC spot with R_f 0.10 (silica gel, 95:5 $CHCl_3$–MeOH) were combined and evaporated to obtain a yellow solid (162 mg, 47%); mp 117–118 °C. The product was recrystallized from MeCN and dried in vacuo over P_2O_5 at 60 °C: TLC R_f 0.70 (silica gel, 9:1 $CHCl_3$–MeOH), 0.48 (5:5:1 $CHCl_3$–MeCN–MeOH); UV: λ_{max} (95% EtOH) 247.5, 285, 342 nm; IR (KBr) 3450, 1740, 1735 (ester C=O), 1640 (amide C=O), 1610 (aromatic) cm^{-1}. Anal. Calcd for $C_{37}H_{52}N_8O_8$: C, 60.31; H, 7.11; N, 15.21. Found: C, 60.29; H, 7.25; N, 14.96.

B. Hexa-*tert*-butyl *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (9d). A mixture of **8d** (474 mg, 0.373 mmol) and acetamidine acetate (176 mg, 1.49 mmol)

in 2-methoxyethanol (15 mL) was heated under reflux for a total of 2 h. The progress of the reaction was monitored by TLC (silica gel, 9:1 $CHCl_3$–MeOH; R_f 0.38 for **9d**, 0.71 for **8d**), and every 20 min another 176-mg portion of acetamidine acetate was added for a total of 1.06 g (8.95 mmol, six additions, 24 mol equiv). After 2 h, the solvent was evaporated, the residue was redissolved in $CHCl_3$ (50 mL), and the solution was washed with H_2O, dried, concentrated to a small volume, and applied onto a silica gel column (40-μm particle size, 29 g, 2 × 28 cm) prepared with 97:3 $CHCl_3$–MeOH. The column was eluted with 250 mL of 97:3 $CHCl_3$–MeOH and then 335 mL of 95:5 $CHCl_3$–MeOH. Evaporation of the first 135 mL of 95:5 eluent gave unchanged **8d** (80 mg, 17% recovery). Evaporation of the following 200 mL of 95:5 eluent afforded **9d** (311 mg, 64%); TLC R_f 0.38. The product was recrystallized from 10 mL of MeCN at −20 °C to obtain pale yellow crystals (202 mg) after drying at 25 °C over P_2O_5; mp 117–119 °C; IR (KBr) 3300, 2980, 2930, 1735 (ester C=O), 1645 (amide C=O), 1610 (aromatic), 1545, 1515, 1450, 1420, 1395, 1370, 1300, 1255, 1160, 1035, 970, 850 cm^{-1}. Anal. Calcd for $C_{64}H_{97}N_{11}O_{17}·H_2O$: C, 58.65; H, 7.61; N, 11.76. Found: C, 58.34; H, 7.39; N, 11.76.

C. Tetra-*tert*-butyl *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (9b): two acetamidine acetate additions, 10 mol equiv; purification by flash chromatography on a silica gel column (2 × 16 cm) with 9:1 $CHCl_3$–MeOH as the eluent; pale yellow solid (62% yield); mp 96–98 °C; TLC R_f 0.40 (silica gel, 9:1 $CHCl_3$–MeOH). Anal. Calcd for $C_{46}H_{67}N_9O_{11}·H_2O$: C, 58.77; H, 7.40; N, 13.41. Found: C, 58.91; H, 7.18; N, 13.46.

D. Penta-*tert*-butyl *N*-[4-[[(4-amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamate (9c): six acetamidine additions, 12 mol equiv; purification similar to that of **9d**; pale yellow soldi (59% yield); mp 118–120 °C; TLC R_f 0.42 (silica gel, 9:1 $CHCl_3$–MeOH). Anal. Calcd for $C_{55}H_{82}N_{10}O_{14}·H_2O$: C, 58.70; H, 7.52; N, 12.45. Found: C, 58.67; H, 7.51; N, 12.36.

Hydrolysis of *tert*-Butyl Esters. A. *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-L-glutamic Acid (7a). A solution of **9a** (162 mg, 0.22 mmol) in a 1:2 mixture of trifluoroacetic acid and CH_2Cl_2 was left to stand at room temperature for 3 h, and the progress of acidolysis was followed by HPLC (see the following text). The solution was poured into a mixture of 5% NH_4OH (20 mL) and $CHCl_3$ (50 mL). The aqueous phase was separated, concentrated to a small volume under reduced pressure, and acidified to pH 4.5 with 10% AcOH. The precipitate was kept at 5 °C overnight and was then centrifuged, washed with a little H_2O, and dried on a lyophilizer to obtain a yellow solid (110 mg): analytical HPLC main peak 5.3 min (25% MeCN in 0.1 M NH_4OAc, pH 6.5, 0.5 mL/min). The product was dissolved in preparative HPLC buffer (20% MeCN in 0.1 M NH_4OAc, pH 7.5) and injected in 20–30-mg portions onto a Dynamax Macro C_{18} column (21.4 mm diameter × 25 cm) that was eluted with the same buffer at a rate of 3.0 mL/min. Fractions containing the main product were pooled, checked by analytical HPLC (>99% purity), evaporated, freeze-dried to constant weight, and kept under vacuum at 60 °C over P_2O_5 to obtain a yellow powder (56 mg, 31%); mp dec from 158 °C. For microanalysis, the powder was dissolved in H_2O (2 mL) and the pH was adjusted to 8.9 with 10% NH_4OH. The insoluble material was centrifuged and the yellow solution decanted and acidified to pH 3.8 with 10% AcOH. The flocculent yellow-orange precipitate was left to stand at 0 °C for 1 h and was then filtered, washed several times with H_2O, and dried, first on a lyphilizer and finally in vacuo at 60 °C over P_2O_5: final yield 33 mg (24%); mp dec from 158 °C; UV λ_{max} (0.1 N NaOH) 221 nm (ε 7900), 246 (15 320), 292 (18 190); λ_{max} (0.1 N HCl) 228 nm (ε 20 090), 237 infl (16 170), 294 (19 115), 330 infl (10 050), 345 infl (7600); MS (FAB) calcd for M + 1, 569; found, 569. Anal. Calcd for $C_{25}H_{28}N_5O_8·2.5H_2O$: C, 48.93; H, 5.42; N, 18.26. Found: C, 48.84; H, 5.36; N, 17.85.

B. *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-L-glutamic Acid (7b). A solution of **8b** (55 mg, 0.059 mmol) in 1 mL of a 1:2 mixture of trifluoroacetic acid and CH_2Cl_2 was kept for 1.25 h at 25 °C and evaporated to dryness in vacuo at room temperature. The residue was taken up in MeOH, excess 28% NH_4OH was added, and the solution was evaporated. The residue was purified by preparative HPLC (C_{18} silica gel, 3% MeCN in 0.1 M NH_4OAc, pH 7.0, 7.0 mL/min). Eluates containing the main peak (17 min) were pooled, evaporated, and freeze-dried for several days with occasional addition of H_2O. When constant weight was reached, the product was dried in vacuo over P_2O_5 at 60 °C to obtain a yellow powder (21 mg, 45%): mp dec from 165 °C; analytical HPLC 28 min (25% MeCN in 0.1 M NH_4OAc, pH 7.0, 2.0 mL/min); IR (KBr) 3430, 2930, 1640, 1610 cm^{-1}; UV λ_{max} (0.1 N NaOH) 247 nm (ε 23 800); 287 (22 800), 340 (8100); λ_{max} (0.1 N HCl) 238 infl (ε 17 600), 294 (19 400), 332 infl (10 800), 345 infl (9000). Anal. Calcd for $C_{30}H_{35}N_9O_{11}·NH_3·4.5H_2O$: C, 45.28; H, 5.95; N, 17.60. Found: C, 45.30; H, 5.55; N, 17.65.

C. *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamic Acid (7c). A solution of **8c** (179 mg, 0.159 mmol) in a 1:2 mixture of trifluoroacetic acid and CH_2Cl_2 (4 mL) was kept at 25 °C for 1.5 h and worked up as in the preceding experiment. Preparative HPLC fractions containing the main product (13.5 min) were pooled, evaporated, freeze-dried to constant weight, and dried over P_2O_5 at 60 °C to obtain a yellow powder (82 mg). The product was dissolved in water (1 mL), and the solution was passed through a 0.45-μm microfilter, diluted to 5 mL, and acidified to pH 3.5 with 10% AcOH. The precipitated solid was centrifuged and freeze-dried to obtain a yellow powder (51 mg, 36%); mp dec from 157 °C; TLC R_f 0.95 (cellulose, pH 7.4 phosphate buffer); analytical HPLC 6.3 min (3% MeCN in 0.1 M NH_4OAc, pH 7.0, 2.0 mL/min); UV λ_{max} (0.1 N NaOH) 248 nm (ε 23 600), 285 (22 800), 340 (8200); λ_{max} (0.1 N HCl) 238 infl (ε 17 000), 294 (19 500), 332 infl (10 550), 345 infl (8400); MS (negative FAB) calcd for M − 1, 826; found, 826. Anal. Calcd for $C_{35}H_{42}N_{10}O_{14}·3H_2O$: C, 47.72; H, 5.49; N, 15.90. Found: C, 47.40; H, 5.25; N, 16.02.

D. *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl-L-glutamic Acid (7d). A solution of **8d** (160 mg, 0.122 mmol) in a 1:2 mixture of trifluoroacetic acid and CH_2Cl_2 (4 mL) was kept at 25 °C for 2 h and worked up as for the other polyglutamates except that the preparative HPLC flow rate was 5 mL/min. Eluates containing the main peak (16 min) were combined, partly evaporated, and freeze-dried for several days, with occasional redissolution in H_2O, until a constant weight (86 mg) was obtained. The product was redissolved in H_2O (2.5 mL), and the solution was acidified to pH 3.5 with 10% AcOH. The solution was applied onto a Biogel P-2 size-exclusion column (2.5 × 82 cm), which was eluted with H_2O. The first yellow band was collected and freeze-dried to constant weight to obtain a yellow powder (65 mg, 49%): mp dec from 117 °C; analytical HPLC 6.1 min (3% MeCN in 0.1 M NH_4OAc, pH 7.0, 1.0 mL/min); IR (KBr) 3420, 3140, 1640, 1610 cm^{-1}; UV: λ_{max} (0.1 N NaOH) 248 nm (ε 30 900), 286 (29 000), 340 (10 400); λ_{max} (0.1 N HCl) 238 nm infl (ε 16 800), 292 (16 500), 332 infl (9550), 345 infl (7500); MS (negative FAB) calcd for M − 1, 955; found, 955. Anal. Calcd for $C_{40}H_{49}N_{11}O_{17}·6H_2O$: C, 45.15; H, 5.68; N, 14.48. Found: C, 45.00; H, 5.28; N, 14.13.

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Registry No. **7a**, 133042-58-7; **7b**·NH_3, 133042-59-8; **7c**, 133042-60-1; **7d**, 133042-61-2; **8a**, 133042-47-4; **8b**, 133042-48-5; **8c**, 133042-49-6; **8d**, 133042-50-9; **9a**, 133042-54-3; **9b**, 133042-55-4; **9c**, 133042-56-5; **9d**, 133042-57-6; **10a**, 88050-24-2; **10b**, 117559-42-9; **10c**, 117559-43-0; **10d**, 117559-44-1; **11**, 133042-41-8; **12**, 133042-42-9; **13a**, 133042-43-0; **13b**, 133042-44-1; **13c**, 133042-45-2; **13d**, 133042-46-3; **14b**, 133042-51-0; **14c**, 133042-52-1; **14d**, 133042-53-2; **15a**, 88063-78-9; **15b**, 73167-55-2; **15b** (*N*-Cbz derivative), 32719-57-6; **15c**, 88443-03-2; **15c** (*N*-Cbz derivative), 32816-47-0; **15d**, 73167-56-3; **15d** (*N*-Cbz derivative), 32719-58-7; Cbz-Glu-OBu-*t*, 5891-45-2; Cbz-Glu(OBu-*t*)-OBu-*t*, 16881-41-7; H-Glu(OMe)-OBu-*t*, 79640-72-5; 4-$O_2NC_6H_4COCl$, 122-04-3; H-Glu(OBu-*t*)-OBu-*t*·HCl, 32677-01-3; $CH_3C(NH_2)$=NH·AcOH, 36896-17-0; 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile, 40127-91-1.