First Completely Chemical Synthesis of [(6*S*)-*N*[5]-Formyltetrahydropteroyl]poly-γ-L-glutamic Acid Derivatives†

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The synthesis of (6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl-di-, -tri-, -tetra- and -penta-γ-L-glutamic acid derivatives **9a–d** has been achieved for the first time through a three-step sequence of chemical reactions. The process involves converting [(6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl]-mono-L-glutamic acid **6** into the α-benzyl monoester **7**, which is then coupled in four separate reactions to preformed polybenzyl esters of mono-, di-, tri-, and tetra-γ-L-glutamate. The resulting (6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl-di-, -tri-, -tetra- and -penta-γ-L-glutamate polybenzyl ester derivatives **8a–d** are then subjected to hydrogenolysis, giving polyacids **9a–d**. The overall yields for the individual sequences range from 37 to 49%. Importantly, enzymic and chiral chromatographic analyses of compounds **9a–d** indicate that their stereochemical purity exceeds 99%.

Folate derivatives play a key role in the biosynthesis of DNA, RNA and certain amino acids. Consequently, they are an essential part of normal cell growth and maturation. As shown in Scheme 1, they are formed from folic acid **1** through a sequence of reactions that involve poly-γ-L-glutamylation of folic acid **1** to compound(s) **2**, bioreduction of compound(s) **2** to compounds **3**–**4** and one-carbon derivatization of compound(s) **4** to compounds **5a–g**.[1]

Medicinal chemists have exploited the pivotal role that folate derivatives play to design a number of potent agents known as antifolates. Antifolates are antitumour agents that are structurally related to folate derivatives. They either block the conversion of folic acid **1** into compounds **5a–g** or interfere with the action of compounds **5a–g** as cofactors. Methotrexate, trimethoprim, trimetrexate and 5,10-dideazatetrahydrofolic acid are notable examples of such agents.[2–4]

Since most tumour cells biosynthesize DNA/RNA at a higher rate than do normal cells, they are more likely to die following exposure to an antifolate. In the case of normal cells, an important exception are the red and white progenitor cells of the bone marrow. Use of antifolates is profoundly toxic to these cells and leads to bone-marrow suppression. Fortunately, the toxic effects on the bone marrow can be dramatically reduced through the use of either [(6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid or a diastereoisomeric mixture of [(6*S*)- and (6*R*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid (leucovorin) following the introduction of the antifolate agent. The natural 6*S* form of *N*[5]-formyl-5,6,7,8-tetrahydropteroylmono-L-glutamic acid raises the level of compounds **5a–g** within the cell and minimizes any unwanted cytotoxic effects on the bone marrow.[5] The therapeutic benefit of the antifolate/[(6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl]-mono-L-glutamic acid regimen to the cancer patient is that a far greater number of sensitive tumour cells are killed per treatment cycle.

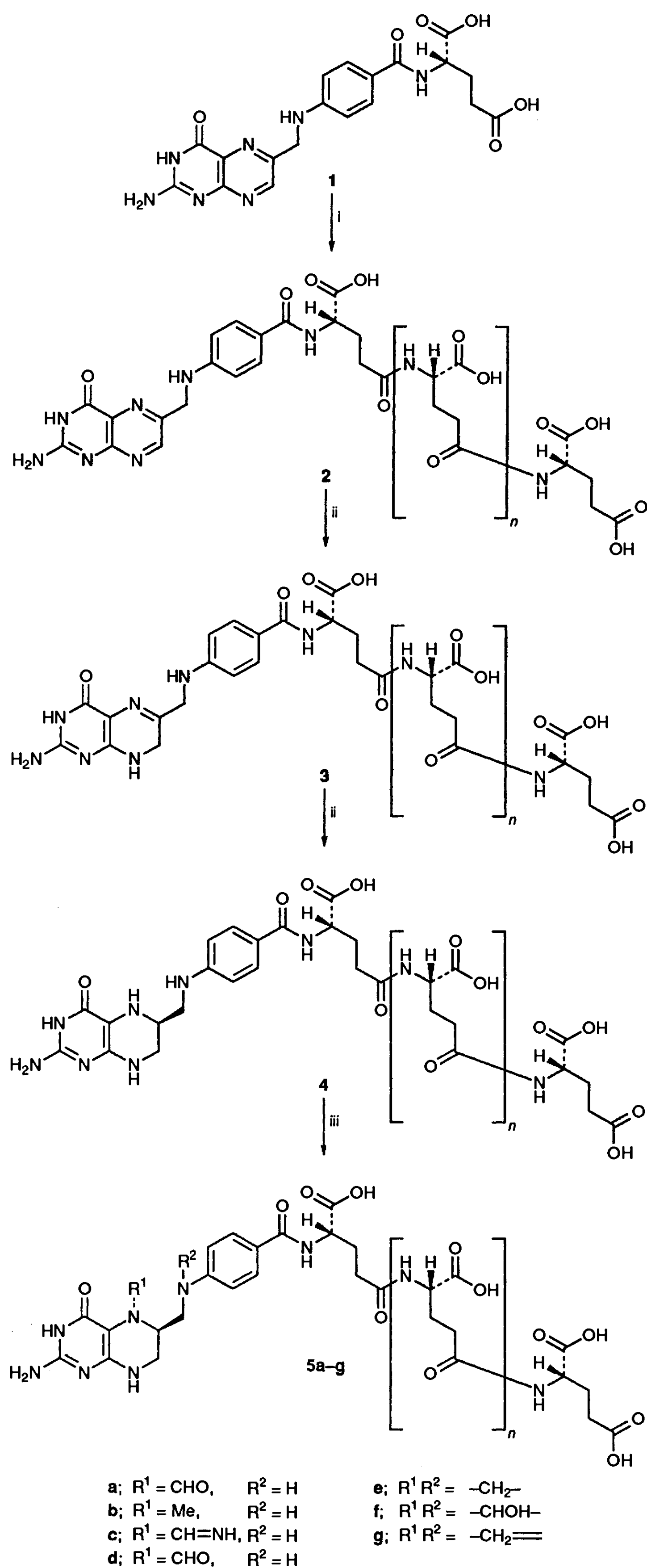
Given the biochemical significance of folate derivatives to cell growth, it is surprising that there are no reports which describe the synthesis of these compounds by wholly chemical means.[6] There are at least three reasons for this omission suggested in the literature. First, in general, 5,6,7,8-tetrahydropteroylpoly-γ-L-glutamic acid derivatives **3**–**5a–g** are extremely sensitive to air or a source of O_2,[7] undergoing rapid autoxidation in the absence of antioxidants like ascorbic acid or 2-mercaptoethanol. Consequently, the laboratory preparation of compounds such as **3**–**5a–g** typically begins with compound **2**, an O_2-stable derivative (in fact, several derivatives of compound **2** are commercially available).[8] Second, there are no known chemical reagents capable of stereospecifically reducing compounds **2** or **3** to **4**. As a result, historically this step has been carried out bioreductively using purified or partially purified dihydrofolate reductase (EC 1.5.1.3) and 1,4-dihydronicotinamide adenine dinucleotide phosphate (NADPH).[8] Third, the poor solubility of most folate derivatives in organic solvents means that protecting groups and deprotecting steps are a common feature of their synthesis.[6] Hence, the prevailing misconception has been that a completely chemical route to compounds **4** or **5a–g** is impractical because it would likely involve a lengthy and expensive reaction sequence.

Herein we describe the chemical synthesis of a set of [(6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl]poly-γ-L-glutamic acid derivatives (**5a**) by way of a three-step sequence. The method is the outgrowth of a preliminary report and patent on the synthesis of (6*S*) and (6*R*) diastereoisomers of *N*[5]-formyl-5,6,7,8-tetrahydropteroyldi-γ-L-glutamic acid.[9,10] It is noteworthy because it represents the first entirely chemical means of preparing biochemical cofactors of this type.

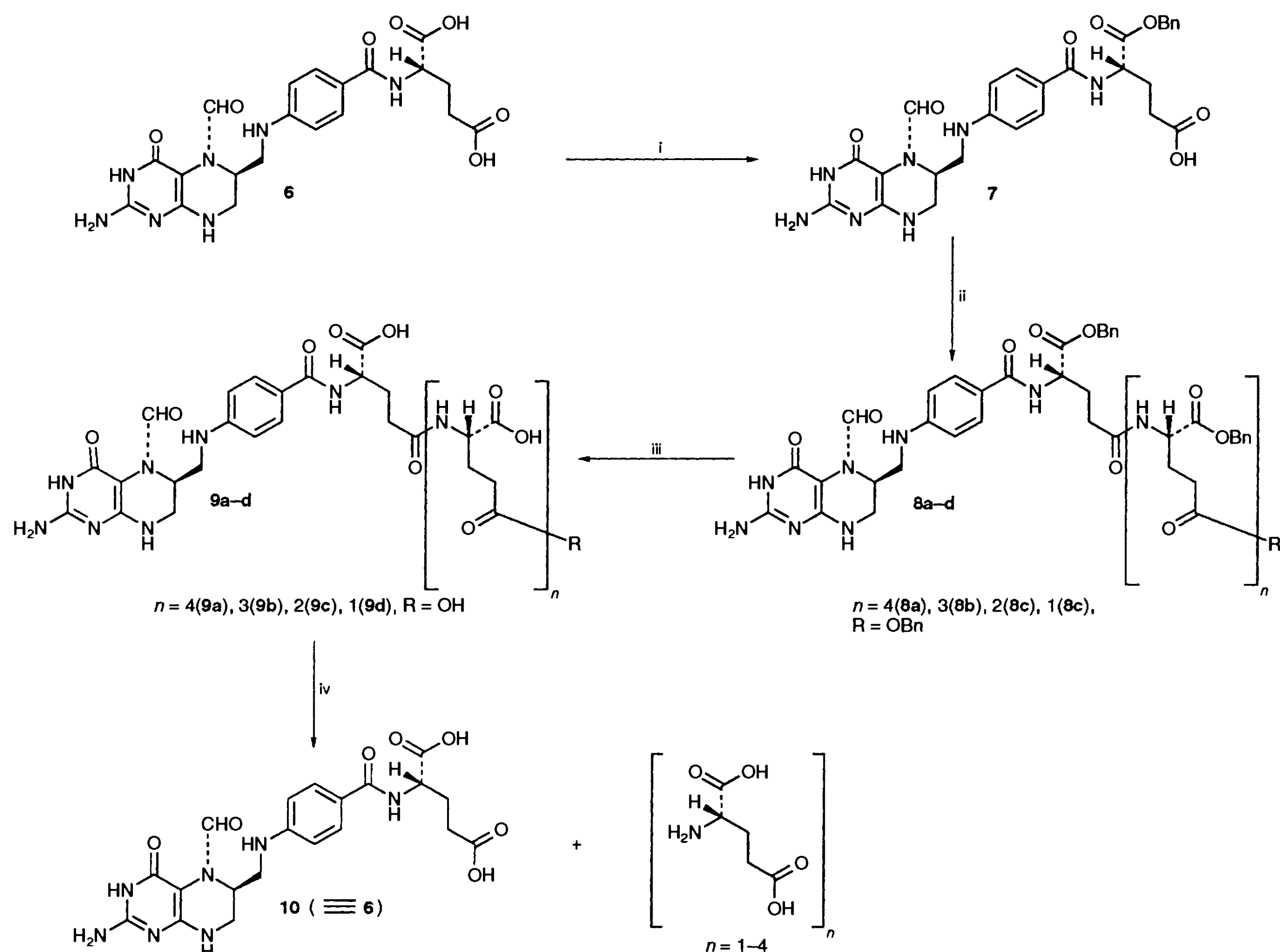
Results and Discussion

In contrast to other strategies,[6] the preparation of polyacids **9a–d** was initiated with the [(6*S*)-*N*[5]-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid derivative because: (i) unlike most reduced folate derivatives, it is stable in the presence of O_2 and therefore manipulatable under normal atmospheric conditions;[7] (ii) already in the naturally occurring (6*S*)-5,6,7,8-tetrahydropteroyl state, it eliminates the need for a biochemical reducing system;[8] and, (iii) it is available in multigram quantities from a commercial source.[11]

† Pteroyl = 4{[(2-amino-3,4-dihydro-4-oxopteridin-6-yl)methyl]-amino}benzoyl.



Scheme 1 *Enzymes and reagents:* i, Pteroylpoly-γ-L-glutamyl synthase, L-glutamic acid; ii, Dihydrofolate reductase (EC 1.5.1.3) NADPH; iii, various enzymes/one-carbon units



Scheme 2 *Reagents, enzymes and conditions:* i, BnBr, $CsCO_3$ in DMSO; ii, Et_3N, $(PhO)_2P(=O)N_3$, and the mono-, di-, tri-, and tetra-γ-L-glutamic acid polybenzyl esters; iii, Pd black, DMF–water (8:2); iv, γ-L-glutamyl hydrolase (EC 3.4.12.10)

The first synthetic step in our three-step sequence involves conversion of [6*S*)-5-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid **6** into monobenzyl ester **7** (Scheme 2). This approach was chosen because Nefkens had earlier shown that under the proper conditions the α-carboxy group of an N-substituted L-glutamic diacid can undergo selective α-derivatization.[12] His method involves reaction of the α,γ-diacid of an N-substituted L-glutamic diacid derivative in dimethylformamide (DMF) with 1 mol equiv. each of base and an alkyl or alkylaryl halide. The lower pK_a of the α-carboxy group causes it to be preferentially ionized. In the presence of an alkylating agent selective derivatization occurs and α-monoesters of N-substituted L-glutamic acids are obtained in good yield. Since compound **6** is an N-substituted L-glutamic diacid derivative, under similar conditions it too should undergo preferential conversion into an α-monoester of [(6*S*)-5-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid **7**. When Nefkens' conditions were rigorously applied to compound **6**, the formation of monoester **7** was not detected. However, because compound **6** remained in suspension during the entire course of the attempted reaction, the failure of Nefkens' method was ascribed to the poor solubility of substrate **6** in DMF. Accordingly, a solvent other than DMF was sought. Rosowsky and Yu[13] reported the solubilization of compound **6** in dimethyl sulfoxide (DMSO) during the synthesis of several α,γ-diesters of [(6*S*)- and (6*R*)-5-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid. Nefkens' procedure was therefore repeated with compound **6** in anhydrous DMSO along with 1.0 mol equiv. of Cs_2CO_3 and 1.1 mol equiv. of benzyl bromide. After 48 h at 23 °C, ester **7** was isolated in 66% yield.*

The synthesis of polyesters **8a–d** was accomplished by attaching the polybenzyl esters of mono- di-, tri-, and tetra-γ-L-glutamate to acid ester **7**. For example, in the preparation of compound **8a**, compound **7** was treated with 2.5 mol equiv. of diphenylphosphoryl azide, 3.0 mol equiv. of triethylamine, and 2.0 mol equiv. of the pentabenzyl ester of tetra-γ-L-glutamate in DMF (first 2 h at 4 °C, followed by 16 h at 25 °C). Purification of the mixture led to the isolation of compound **8a** in near theoretical yield (99%). Catalytic reduction of polyester **8a** over Pd black in aq. DMF [water:DMF in the ratio 8:2 (v/v); 15 h; 25 °C] gave a good yield of polyacid **9a** (75%). In a manner similar to polyacid **9a**, compounds **9b–d** were prepared in overall yields ranging from 37 to 49% or roughly 10-fold higher than those previously attained.

The next problem addressed was the stereochemical purity of polyacids **9a–d**. Initially, the poly-γ-L-glutamic acid 'tail' region of compounds **9a–d** was examined. After Hanlon *et al.*,[14]† small samples of acids **9a–d** were incubated separately with a hog kidney γ-glutamyl hydrolase (EC 3.4.12.10) preparation; this

* Although the yield at this step is modest, the γ-monoester (7%) and α,γ-diester (20%) formed as by-products may be returned to starting material, **6**, *via* catalytic reduction over Pd black. The actual losses of starting material are consequently negligible.

† The crude hog kidney γ-glutamyl hydrolase used in our assay of compounds **10a–d** was the kind gift of Dr. Carmen J. Allegra, NCI.

peptidase specifically cleaves those peptide bonds attached to γ-linked L-glutamate residues. Importantly, it does not hydrolyse α-linked L-, α-linked D-, or γ-linked D-glutamate residues. Since the retention times of compounds **9a–d** were all distinct under the HPLC conditions employed (see Experimental section), the hydrolysis of each derivative could be followed to completion (formation of product **10** + free L-glutamic acid moieties). This method demonstrates unequivocally that the 'tail' region of all of the synthetically prepared [(6*S*)-5-formyl-5,6,7,8-tetrahydropteroyl]poly-γ-L-glutamic acid derivatives **9a–d** have stereochemical purities that exceed 99%. Additionally, the stereochemical purity of the product **10** was subsequently ascertained by injecting small samples of the final hydrolysate onto a chiral albumin-linked silica gel HPLC column.[15] This test confirmed that the starting material, **6**, is identical with compound **10** (*i.e.*, consisting of >99% 6*S* diastereoisomer). The combined use of enzymic and chiral chromatographic techniques indicates that acids **9a–d** have a stereoconfiguration that is identical with that of naturally occurring derivatives of (N^5-formyl-5,6,7,8-tetrahydropteroyl)-poly-γ-L-glutamic acid (**5a**, Scheme 1).

In this paper we describe the first ever chemical synthesis of four naturally occurring [(6*S*)-N^5-formyl-5,6,7,8-tetrahydropteroyl]poly-γ-L-glutamic acid derivatives **9a–d**. It contrasts with previous efforts that require both chemical and biochemical reagents. The stereochemical purity of the products **9a–d** prepared by this approach exceeds 99% and the overall yields of the method are about 10-fold higher than those achieved previously.[6] The method's inherent flexibility should allow it to be adapted to the synthesis of reduced folate derivatives of nearly every type, chain length, or α,γ-composition. In this regard, the method is currently being adapted for use in the preparation of polyacids **4** and **5b–d**. The results of this effort will be reported at a later date.

Experimental

The stereochemically pure form of [(6*S*)-5-formyl-5,6,7,8-tetrahydropteroyl]mono-L-glutamic acid used in this report was the kind gift of Drs. Joseph E. Dolfini and F. Marazza of Nobilent, Inc., Cincinnati, Ohio 45242 USA and SAPEC S.A. Fine Chemicals, Lugano, Switzerland CH-6903, respectively. All other chemicals were obtained from Fluka Chemical Co., Inc., Ronkonkoma, New York 11779 USA. The C_{18} (4.6 mm ID × 250 mm, Partisil 10 ODS) and albumin-linked silica gel (4.6 mm ID × 250 mm, Resolvosil BSA-7) columns were purchased from Whatman, Inc., Clifton, New Jersey 07014 USA and Alltech Associates, Inc., Deerfield, Illinois 6001 USA, respectively. HPLC analysis was performed on a Hewlett-Packard liquid chromatographic system, Avondale, PA 19311-9990 USA. The components include a model 1050 pumping system with a column heater, a 1050 UV-visible variable-wavelength detector and a 3396A integrator. The purity of the [(6*S*)-N^5-formyl tetrahydropteroyl]poly-γ-L-glutamic acid derivatives were assessed under the following conditions: 25 °C, C_{18} column, (15:85) propan-2-ol–0.005 mol dm^{-3} tetrabutylammonium phosphate, 280 nm detection, and a flow rate of 1 cm^3 min^{-1}. M.p.s were measured on a Fisher–Johns melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Varian VXR-500S spectrometer with a SUN 4/110 workstation. *J* Values are given in Hz. NMR assignment of the partial structure for the protons at positions 6,7,8,9 and 10 was based on chemical-shift comparisons of this area to that in ref. 16. These proton assignments were obtained from the following 2D heteronuclear multiple bond coherence (HMBC) correlations: 7-H^{ab} to C-6, C-8 and C-9; 6-H to 5-CHO and C-9; and 9-H^{ab} to C-4′, C-6 and C-7.* Mass spectra were obtained for all of the (6*S*)-N^5-formyltetrahydropteroyl α-mono and poly-γ-L-glutamate polyester derivatives (except the penta-γ-L-hexaglutamate ester derivative). By contrast, none of the [(6*S*)-N^5-formyl-5,6,7,8-tetrahydropteroyl]poly-γ-L-glutamic acid derivatives gave such spectra. Presumably, the analysis of these derivatives was prevented by their low volatility.

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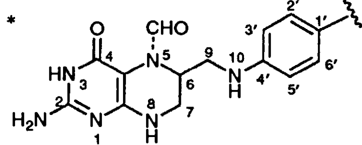

α-Benzyl Ester of [6(S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]-*mono*-L-*glutamic Acid* **7**.—Compound **6** was prepared by modification of a previously described method.[9] Benzyl bromide was substituted for the 2,6-dichlorobenzyl bromide described in the above referenced procedure. Purification on silica gel 60 [$CHCl_3$–MeOH (containing 3% AcOH) (7:1) as eluent], rotary evaporation (40 min at 40 °C, 5 mmHg) followed by drying *in vacuo* (12 h at 40 °C, 0.1 mmHg) gave compound **7** as a pale yellow solid (392 mg, 66%), m.p. > 300 °C (Found: C, 54.5; H, 5.15; N, 15.9. $C_{27}H_{29}N_7O_7$ + 0.85 CH_3CO_2H + 0.85 H_2O requires C, 54.72; H, 5.45, N, 15.56%); ν_{max}(KBr)/cm^{-1} 3340 (NH), 1727 (ester CO), 1620 (amide CO), 1325 (CH), 1188 amide (CO) and 765 (aryl CH); δ_H([$^2H_6$]-DMSO; 500 MHz) 1.96 (1 H, cm, β-H_2), 2.01 (1 H, cm, β-H), 2.19 (2 H, t, *J* 6.5, γ-H_2), 2.94 (1 H, ddd, *J* 5.0, 7.3 and 13.5, 9-H^b), 3.07 (1 H, ddd, *J* 6.9, 7.9 and 13.5, 9-H^a), 3.13 (1 H, dd, *J* 4.4 and 12.3, 7-H^b), 3.38 (1 H, dd, *J* 4.9 and 12.2, 7-H^a), 4.35 (1 H, cm, α-H), 4.78 (1 H, ddd, *J* 4.9, 7.3 and 7.9, 6-H), 5.11 (1 H, AB, *J* 12.6, OCH_2), 5.13 (1 H, AB, *J* 12.7, OCH_2), 6.29 (1 H, dd, *J* 5.0 and 6.9, 10-NH), 6.53 (2 H, d, *J* 8.7, 3′- and 5′-H), 6.59 (2 H, br s, 2-NH_2), 6.93 (H, d, *J* 4.4, 8-H), 7.28–7.38 (5 H, cm, ArH), 7.28–7.38 (1 H, obs. d, 8-NH), 7.62 (2 H^d, *J* 8.5, 2′- and 6′-H), 8.81 (s, 5-CHO), 9.31 (1 H, br s, 3-NH) and 11.12 (1 H, br s, CO_2H); *m*/*z* 564 (MH^+, 9%) and 185 (100%).

General Procedure for the Preparation of Polyesters **8a–d**. *Hexabenzyl Ester of* [(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]-*penta*-γ-L-*glutamic Acid* **8a**.—Compound **7** (200 mg, 0.35 mmol),[9] the tetrabenzyl ester of tetra-γ-L-glutamate hydrochloride salt (680 mg, 0.85 mmol), triethylamine (0.149 cm^3, 1.06 mmol), and diphenylphosphoryl azide (0.192 cm^3, 0.89 mmol) were added to anhydrous DMF (20 cm^3) in a manner essentially identical with that of Piper *et al.*[16] Work-up proceeded as follows: excess of DMF was removed on a rotary evaporator (3 h at 40 °C, 5 mmHg) and the remaining oily residue was treated with ice-cold water (15 cm^3) whereupon a precipitate formed. The precipitate was collected *via* centrifugation (8000 rpm at 4 °C for 20 min) and the supernatant was decanted. The precipitate was washed twice more with ice-cold water (10 cm^3) and freeze-dried overnight (16 h). Purification of the freeze-dried solid on a silica gel 60 column [$CHCl_3$–MeOH (9:1) as eluent] gave compound **8a** in near theoretical yield (464 mg, 99%), m.p. 170–172 °C (Found: C 62.7; H, 5.8; N, 9.15. $C_{82}H_{87}N_{11}O_{19}$ + 3.5 CH_3OH + 0.25 H_2O requires C, 62.47; H, 6.01; N, 9.37%); ν_{max}(KBr)/cm^{-1} 3300 (NH), 1728 (ester CO), 1645 (amide CO), 1335 (CH), 1182 (amide CO) and 764 (aryl CH); δ_H([$^2H_6$]DMSO; 500 MHz) 1.70–2.60 (20 H, cm, CH_2), 2.84–2.90 (1 H, cm, 9-H^b), 3.00–3.20 (2 H, cm, 9-H^a and 7-H^b), 3.40 (1 H, cm, 7-H^a), 4.29 (4 H, cm, α-H), 4.46 (1 H, cm, α-H), 4.80 (1 H, cm, 6-H), 5.09 (12 H, cm, OCH_2), 6.21 (2 H, br s, 2-NH_2), 6.37 (1 H, X of ABX, 10-NH), 6.61 (2H, d, *J* 8.3, 3′- and 5′-H), 7.00 (1 H, X of ABX, 8-NH), 7.32 (30 H, cm, ArH), 7.69 (2 H, d, *J* 8.5, 2′- and 6′-H), 8.25 (1 H, d, *J* 7.3, NHCO), 8.25 (1 H, d, *J* 7.3, NHCO), 8.32 (1 H, d, *J* 7.4, NHCO), 8.35 (1 H, d, *J* 7.8, NHCO), 8.40 (1 H, d, *J* 7.1, NHCO), 8.85 (1 H, s, 5-CHO) and 10.24 (1 H, br s, 3-NH); *m*/*z* not obtainable.

Pentabenzyl Ester of [(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]*tetra*-γ-L-*glutamic Acid* **8b**.—89%; m.p. 151.5–152.5 °C (Found: C, 62.5; H, 6.0; N, 10.2. $C_{70}H_{74}N_{10}O_{16}$ + 1.0 CH_3OH + 1.0 H_2O requires C, 62.64; H, 5.92; N, 10.29%); ν_{max}(KBr)/cm^{-1} 3300 (NH), 1730 (ester CO), 1640 (amide CO), 1326 (CH), 1190 (amide CO) and 751 (aryl CH); δ_H([$^2H_6$]-DMSO; 500 MHz) 1.70–2.50 (16 H, cm, CH_2), 2.87 (1 H, td, *J* 5.5 and 13.5, 9-H^b), 3.06 (1 H, ddd, *J* 6.7, 8.6 and 13.5, 9-H^a), 3.13 (1 H, dd, *J* 4.2 and 12.6, 7-H^b), 3.40 (1 H, dd, *J* 5.0 and 12.2, 7-H^a), 4.23–4.34 (3 H, 2 cm, α-H), 4.46 (1 H, ddd, *J* 4.6, 7.6 and 10.1, α-H), 4.80 (1 H, cm, 6-H), 5.06 (2 H, AB, *J* 12.8, OCH_2), 5.08 (3 H, AB, *J* 12.7, OCH_2), 5.10 (3 H, AB, *J* 12.7, OCH_2), 5.12 (2 H, AB, *J* 12.7, OCH_2), 6.12 (2 H, br s, 2-NH_2), 6.36 (1 H, X of ABX, 10-NH), 6.61 (2 H, d, *J* 8.9, 3'- and 5'-H), 6.98 (1 H, X of ABX, 8-NH), 7.27–7.37 (25 H cm, ArH), 7.70 (2 H, d, *J* 8.8, 2'- and 6'-H), 8.25 (1 H, d, *J* 7.4, NHCO), 8.28 (1 H, d, *J* 7.5, NHCO), 8.34 (1 H, d, *J* 7.5, NHCO), 8.39 (1 H, d, *J* 7.3, NHCO), 8.85 (1 H, s, 5-CHO) and 10.26 (1 H, s, 3-NH); *m/z* 1312 (MH^+, 14%) and 327 (100%).

Tetrabenzyl Ester of [(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]*tri*-γ-L-*glutamic Acid* **8c**.—Ethanol was substituted for methanol during the purification; 87%; m.p. 105–108 °C (Found: C, 62.7; H, 5.9; N, 10.5. $C_{58}H_{61}N_9O_{13}$ + 2.0 C_2H_5OH requires C, 62.73; H, 6.21; N, 10.64%); ν_{max}(KBr)/cm^{-1} 3325 (NH), 1734 (ester CO), 1635 (amide CO), 1323 (CH), 1185 (amide CO) and 760 (aryl CH); δ_H[$^2H_6$]DMSO; 500 MHz) 1.70–2.50 (12 H, cm, CH_2), 2.86 (1 H, td, *J* 5.4 and 13.3, 9-H^b), 3.06 (1 H, ddd, *J* 6.5, 8.2 and 13.3, 9-H^a), 3.12 (1 H, dd, *J* 4.1 and 12.6, 7-H^b), 3.40 (1 H, dd *J* 4.8 and 12.6, 7-H^a), 4.27 (1 H, ddd, *J* 5.2, 7.5 and 9.4, C*H*NH), 4.31 (1 H, ddd, *J* 5.4, 7.6 and 9.0, C*H*NH), 4.45 (1 H, ddd, *J* 4.6, 7.4 and 10.1, C*H*NH), 4.80 (1 H, cm, 6-H), 5.06 (2 H, AB, *J* 12–13, OCH_2), 5.09 (2 H, AB, *J* 12–13, OCH_2), 5.09 (2 H, AB, *J* 12–13, OCH_2), 5.12 (2 H, AB, *J* 12–13, OCH_2), 6.24 (2 H, br s, 2-NH_2), 6.36 (1 H, X of ABX, 10-NH), 6.61 (2 H, d, *J* 8.7, 3'-and 5'H), 6.97 (1 H, X of ABX, 8-NH), 7.33 (20 H, cm, ArH), 7.69 (2 H, d, *J* 8.8, 2'- and 6'-H), 8.29 (1 H, d, *J* 7.4, NHCO), 8.30 (1 H, d, *J* 7.4, NHCO), 8.39 (1 H, d, *J* 7.6, NHCO), 8.85 (1 H, s, 5-CHO) and 10.29 (1 H, br s, 3-NH); *m/z* 1093 (MH^+, 20%) and 233 (100%).

Tribenzyl Ester of [(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]-*di*-γ-L-*glutamic Acid* **8d**.—Ethanol was substituted for methanol during the purification; 88%; m.p. 105–108 °C (Found: C, 62.5; H, 6.15; N, 11.6. $C_{46}H_{48}N_8O_{10}$ + 2.0 C_2H_5OH requires C, 62.23; H, 6.27; N, 11.61%); ν_{max}(KBr)/cm^{-1} 3325 (NH), 1730 (ester CO), 1630 (amide CO), 1323 (CH), 1185 amide (CO) and 765 aryl (CH); δ_H([$^2H_6$]DMSO; 500 MHz) 1.84 (cm, 1 H, CH_2), 1.96 (1 H, cm, CH_2), 2.00 (1 H, cm, CH_2), 2.10 (1 H, cm, CH_2), 2.28 (2 H, cm, CH_2), 2.42 (2 H, cm, CH_2), 2.86 (1 H, td, *J* 5.4 and 13.3, 9-H^b), 3.06 (1 H, ddd, *J* 6.7, 8.8 and 13.3, 9-H^a), 3.13 (1 H, dd, *J* 4.2 and 12.6, 7-H^b), 3.40 (1 H, dd, *J* 5.2 and 12.3, 7-H^a), 4.32 (1 H, ddd, *J* 5.3, 7.4 and 9.1, α-H), 4.43 (1 H, ddd, *J* 4.8, 7.4 and 10.0, α-H), 4.80 (1 H, cm, 6-H), 5.07 (1 H, AB, *J* 12.9, OCH_2), 5.07 (1 H, AB, *J* 12.9, OCH_2), 5.09 (1 H, AB, *J* 12.6, OCH_2), 5.10 (1 H, AB, *J* 12.7, OCH_2), 5.13 (1 H, AB, *J* 12.8, OCH_2), 5.13 (1 H, AB, *J* 12.8, OCH_2), 6.23 (2 H, br s, 2-NH_2), 6.35 (1 H, X of ABX, 10-NH), 6.60 (2 H, d, *J* 8.8, 3'- and 5'-H), 6.97 (1 H, X of ABX, 8-NH), 7.33 (15 H, cm, ArH), 7.68 (2 H, d, *J* 8.8, 2'- and 6'-H), 8.31 (1 H, d, *J* 7.4, NHCO), 8.36 (1 H, d, *J* 7.5, NHCO), 8.85 (1 H, s, 5-CHO) and 10.28 (1 H, br s, H); *m/z* 873 (MH^+, 14%) and 119 (100%).

General Procedure for the Preparation of Acids **9a–d**. [(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]*penta*-γ-L-*glutamic Acid* **9a**.—Compound **8a** (186 mg, 0.14 mmol) and Pd black (200 mg) were added to a DMF–water (8:2) mixture (10 cm^3). The suspension was carefully degassed and a latex balloon containing H_2 was attached to the reaction flask. After 20 h at room temperature, the suspension was filtered and excess of solvent removed by rotary evaporation (6 h at 30 °C, 5 mmHg). The remaining residue was then purified chromatographically on Cellulose [0.5 mol dm^{-3} aq. NH_4HCO_3–propan-2-ol (1:1)]. Excess of solvent was initially removed on a rotary evaporator (25 °C, 10 mmHg) until the final volume was ~20 cm^3). The suspended product was then freeze-dried overnight (16 h) and dried *in vacuo* (3 h at 50 °C, 0.1 mmHg) to give compound **9a** (122 mg, 75%), m.p. 275–285 °C (decomp., progressive darkening > 155 °C) (Found: C, 41.9; H, 6.2; N, 17.3. $C_{40}H_{48}N_{11}O_{19}$ + $6H_2O$ + $3NH_4^+$ requires C, 41.81; H, 6.32; N, 17.07%); ν_{max}(KBr)/cm^{-1} 3266 (NH, OH), 1636 (amide CO), 1331 (CH), 1188 (amide CO) and 770 (aryl CH); δ_H-[$^2H_6$]DMSO; 500 MHz), 1.66–1.82 (cm, 5 H, CH_2), 1.85–2.02 (4 H, cm, CH_2), 2.02–2.28 (11 H, cm, CH_2), 2.91 (1 H, t_{AB} *J* 5.8 and 13.2, 9-H^b), 3.07 (1 H, dd, *J* 6.6, 7.6 and 13.2, 9-H^a), 3.13 (1 H, d_{AB}, *J* 3.9 and 12.4, 7-H^b), 3.39 (1 H, d_{AB} *J* 4.6 and 12.1, 7-H^a), 4.04 (4 H, cm, α-H), 4.19 (1 H, dt, *J* 5.1 and 7.3, α-H), 4.79 (1 H, dt, *J* 3.7 and 7.1, 6-H), 6.23 (1 H, X of ABX, 10-NH), 6.56 (2 H, d, *J* 8.7, 3'- and 5'-H), 6.62 (2 H, br s, 2-NH_2), 6.99 (1 H, X of ABX, 8-NH), 7.62 (2 H, d, *J* 8.6, 2'- and 6'-H), 7.81 (1 H, d, *J* 7.4, NHCO), 7.83 (1 H, d, *J* 7.4, NHCO), 7.84 (1 H, d, *J* 7.4, NHCO), 7.85 (1 H, d, *J* 7.4, NHCO), 7.92 (1 H, d, *J* 6.7, NHCO), 8.20 (1 vbr s, 3-NH) and 8.79 (1 H, s, 5-CHO); HPLC, single peak at t_R 31.27 min.

[(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]*tetra*-γ-L-*glutamic Acid* **9b**.—70%, m.p. 275–285 °C (decomp., progressive darkening > 160 °C) (Found: C, 43.7; H, 5.6; N, 16.2. $C_{35}H_{43}N_{10}O_{16}$ + 4.5 H_2O + 1.0 NH_4 requires C, 43.98; H, 5.59; N 16.12%); ν_{max}(KBr)/cm^{-1} 3280 (NH, OH), 1630 (amide CO), 1330 (CH), 1190 (amide CO) and 770 (aryl CH); δ_H-([$^2H_6$]DMSO; 500 MHz) 1.72 (2 H, cm, CH_2), 1.78 (2 H, cm, CH_2), 1.90 (2 H, cm, CH_2), 1.96 (2 H, cm, CH_2), 2.12 (4 H, cm, CH_2), 2.20 (4 H, cm, CH_2), 2.92 (1 H, t_{AB}, *J* 6.1 and 13.5, 9-H^b), 3.06 (1 H, t_{AB}, *J* 6.8 and 14.1, 9-H^a), 3.13 (1 H, d_{AB} *J* 4.1 and 12.4, 7-H^b), 3.39 (1 H, d_{AB} *J* 4.9 and 12.3, 7-H^a), 4.04 (2 H, dt *J* 4.7 and 8.1, α-H), 4.09 (1 H, q, *J* 7.3, α-H), 4.21 (1 H, dt, *J* 5.0 and 7.8, α-H), 4.79 (1 H, dt, *J* 3.9 and 7.2, 6-H), 6.21 (1 H, X of ABX, 10-NH), 6.56 (2H, d, *J* 8.8, 3'- and 5'-H), 6.56 (2 H, s, 2-NH_2), 6.95 (1 H, X of ABX, 8-NH), 7.63 (2 H, d, *J* 8.7, 2'- and 6'-H), 7.82 (2 H, d, *J* 7.6, NHCO), 7.85 (1 H, d, *J* 7.7, NHCO), 7.97 (1 H, d, *J* 7.1, NHCO) and 8.80 (1 H, s, 5-CHO); HPLC, single peak at t_R 21.11 min.

[(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]*tri*-γ-L-*glutamic Acid* **9c**.—65%; m.p. 250–260 °C [decomp., progressive darkening > 175 °C (yellow)] (Found: C, 42.35; H, 5.9; N, 16.0. Calc. for $C_{30}H_{37}N_9O_{13}$ + 6 H_2O + 0.5 NH_4. C, 42.45; H, 6.06; N, 15.68%); ν_{max}(KBr)/cm^{-1} 3260 (NH, OH), 1620 (amide CO), 1325 (CH), 1190 (amide CO) and 770 (aryl CH); δ_H-([$^2H_6$]DMSO; 500 MHz) 1.60–2.30 (12 H, cm, CH_2), 2.92 (1 H, td, *J* 6.0 and 13.4, 9-H^b), 3.07 (1 H, td, *J* 6.9 and 13.4, 9-H^a), 3.13 (1 H, dd, *J* 4.2 and 12.6, 7-H^b), 3.39 (1 H, dd, *J* 5.0 and 12.6, 7-H^a), 4.02 (1 H, dt, *J* 4.9 and 8.1, α-H), 4.07 (1 H, q, *J* 7.3, α-H), 4.15 (1 H, dt *J* 5.6 and 6.6, α-H), 4.79 (1 H, dt, *J* 4.2 and 6.9, 6-H), 6.19 (1 H, X of ABX, 10-NH), 6.51 (2 H, br s, 2-NH_2), 6.56 (2 H, d, *J* 8.6, 3'- and 5'-H), 6.94 (1 H, X of ABX, 8-NH), 7.60 (2 H, d, *J* 8.5, 2'- and 6'-H), 7.72 (1 H, br d, *J* 6.3, NHCO), 7.78 (1 H, cm, NHCO), 7.89 (1 H, cm, NHCO), 8.80 (1 H, s, 5-CHO) and 9.87 (1 H, br s, 3-NH); HPLC, single peak at t_R 14.95.

[(6S)-5-*Formyl*-5,6,7,8-*tetrahydropteroyl*]*di*-γ-L-*glutamic Acid* **9d**.—77%, m.p. 250–260 °C [decomp., progressive darkening > 180 °C (yellow)] (Found: C, 44.6, H, 5.4; N, 17.3. $C_{25}H_{30}N_8O_{10}$ + 3.50 H_2O + 0.5 NH_4; C, 44.8; H, 5.71; N, 17.24%); ν_{max}(KBr)/cm^{-1} 3230 (NH, OH), 1620 (amide

CO), 1320 (CH), 1185 (amide CO) and 800 (aryl CH); δ_H([$^2H_6$]DMSO; 500 MHz) 1.70 (1 H, cm, CH_2), 1.80 (1 H, cm, CH_2), 1.90 (1 H, cm, CH_2), 2.00 (1 H, cm, CH_2), 2.20 (3 H, cm, CH_2), 2.27 (1 H, cm, CH_2), 2.88 (1 H, td, *J* 6.5 and 13.6, 9-H^b), 3.07 (1 H, ddd, *J* 6.9, 8.1 and 13.6, 9-H^a), 3.13 (1 H, dd, *J* 4.2 and 12.7, 7-H^b), 3.40 (1 H, dd, *J* 4.9 and 12.5, 7-H^a), 4.14 (1 H, q, *J* 7.6, α-H), 4.19 (1 H, dt, *J* 4.8 and 7.6, α-H), 4.79 (1 H, cm, 6-H), 6.28 (2 H, br s, 2-NH_2), 6.30 (1 H, X of ABX, 10-NH), 6.59 (2 H, d, *J* 8.8, 3'- and 5'-H), 6.96 (1 H, X of ABX, 8-NH), 7.63 (2 H, d, *J* 8.7, 2'- and 6'-H), 7.74 (1 H, d, *J* 7.2, NHCO), 8.02 (1 H, d, *J* 6.3, NHCO) and 8.83 (1 H, s, 5-CHO); HPLC, single peak at t_R 10.08 min.

Enzymic and Chiral HPLC Analyses of Acids **9a–d**.—The digestion of the terminal poly-γ-glutamate residues of acids **9a–d** were conducted as per the method of Hanlon *et al.*,[14] The formation of compound **10** (≡ **6**) (HPLC retention time t_r 8.61 min) was followed by periodically injecting small samples of the hydrolysate onto a C_{18} column. After the hydrolysis of acids **9a–d** to diacid **10** was complete, its chiral purity was ascertained on an albumin-linked silica gel column.[15] In all cases, the stereochemical purity of diacid **10** exceeded 99%.

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