Supplementary Information

Synthesis of (6R)- and (6S)-5,10-Dideazatetrahydrofolate Oligo-γ-glutamates: Kinetics of Multiple Glutamate Ligations Catalyzed by Folylpoly-γ-glutamate Synthetase

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Results

The synthesis of 6RS-7 is illustrated in Scheme S1.1,2 Malonaldehyde bis(dimethyl acetal) (S1) was treated with aqueous HCl followed by bromine to yield crude bromomalonaldehyde. This crude material was then treated with aqueous NaOH to form the sodium salt (S2) that was isolated by crystallization from wet acetone.

6-Bromo-5-deazapterin (S4) was formed in an HBr-catalyzed dehydration reaction between S2 and 2,4-diamino-6-hydroxypyrimidine (S3). The literature procedure1 for the synthesis of S4 calls for the use of HCl as the acid. Initial experiments used bromomalonaldehyde sodium salt and an extra equivalent of HCl in a reaction that was much diluted with absolute EtOH to allow stirring. These reaction conditions resulted in a 1 to 0.3 mixture (as determined by 1H NMR) of bromo- to chloro-containing products. The identity of these products was confirmed by HRMS (324.0212, required for C12H13N4O2Br 324.02219; 280.0724, required for C12H13N4O2Cl 280.07270; the relative isotopic abundance patterns also supported this assignment) of the 2-pivaloated mixture. Only the desired product was obtained when the acid was changed to 30% HBr in acetic acid. The exocyclic amine of S4 was then protected by reaction with pivaloyl chloride in pyridine to yield 2-pivaloyl-6-bromo-5-deazapterin (S5). The general procedure followed was that reported for 2-pivaloylguanine by Taylor.3 Pivaloyl anhydride may also be used to effect this conversion.1

Methyl 4-vinylbenzoate (S7) was prepared from 4-vinylbenzoic acid (S6) by the method of Webb4 where anhydrous methanol and conc. H2SO4 were used to form the methyl ester. Formation of S7 was also achieved by the use of thionyl chloride5 in place of conc. H2SO4, but the results of this

Scheme S1 The synthesis of 6RS-7.
method were less reproducible. In both cases, the reaction was run at a high dilution (1 g per 100 mL) to minimize acid-catalyzed polymerization reactions. Methyl 4-vinylbenzoate (S7) was reported by Taylor et al. to be accessible by diazomethane methylation of 4-vinylbenzoic acid. In our hands, this reaction proved to be incompatible with the vinyl functionality.

The formation of methyl 2-pivaloyl-5,10-dideoxa-9,10-dehydropteroylate (S8) was accomplished by coupling S5 and S7 with a standard Heck reaction. This product, S8, was then reduced by catalytic hydrogenation with 10% Pd/C and 50 psi H2 in a 1:4 mixture of MeOH:THF to yield methyl (6R)-2-pivaloyl-5,10-dideoxa-9,7,8-tetrahydropteroylate, (S6S,7). Reduction of S8 to 6RS-7 was reported by Taylor and was carried out at ~8 mg/mL in MeOH and was limited by the solubility of S8. With a 1:4 mixture of MeOH:THF, the solubility of S8 was improved and the reduction could be done at much higher concentrations. Multiple rounds of reduction with fresh catalyst were necessary as a result of loss of catalyst activity as the reduction proceeded. The racemic product, 6RS-7, may then be carried on directly to give (6R)-DDAH4PteGlu1 (Scheme 2). Alternatively, the individual isomers, 6R- or 6S-7, may be separated by chiral HPLC (Figures S1, S2) and converted to stereochemically pure (6R)- or (6S)-DDAH4PteGlu1 (Scheme 2) and the poly(-glutamyl derivatives (Scheme 3).

Experimental

Materials. As in main text except for as indicated below. Malonaldehyde bis(dimethyl acetal) and 2,4-diamino-6-hydroxy-3,7-pyrimidinone were obtained from Aldrich. Pd(OAc)2 and tri-o-tolyolphosphine were obtained from Strem Chemicals.

General Procedures. As in the main text except for as described below.

Semi-preparative reversed phase(RP)-HPLC (Method 1S): Column - Varian Dynamax 21.4 x 250 mm, Microsorb 60-8, C18. Flow rate of 10 mL/min. Eluant A - 0.1% v/v TFA in dH2O. Eluant B - 0.1% w/v TFA in CH3CN. Detection at 214 nm. Gradient - 0 min, 1% B; 5 min, 1% B; 15 min, 5% B; 20 min, 100% B.

Bromomalonaldehyde.7 Malonaldehyde bis(dimethyl acetal) (S1) (100.00 g, 0.609 mol) was added with stirring to 100 mL dH2O. Concentrated HCl (4.33 mL, 0.052 mol) was added converted to stereochemically pure (6S,7). Alternatively, the individual isomers, 6R- or 6S-7, may be separated by chiral HPLC (Figures S1, S2) and converted to stereochemically pure (6R)- or (6S)-DDAH4PteGlu1 (Scheme 2) and the poly(-glutamyl derivatives (Scheme 3).

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Bromomalonaldehyde.7 Malonaldehyde bis(dimethyl acetal) (S1) (100.00 g, 0.609 mol) was added with stirring to 100 mL dH2O. Concentrated HCl (4.33 mL, 0.052 mol) was added slowly and the reaction was stirred for 2 h. The solution was then cooled in an ice-water bath and Br2 was added such that the temperature of the solution remained below 15 °C. The reaction was stirred for a further 30 min on ice. The solution was then concentrated at 35-40 °C and 5 mm Hg until a thick crystalline paste had formed. This was filtered and washed with 200 mL cold water followed by 100 mL cold CH2Cl2. After drying under high vacuum, crude product was recovered. A crude yield of 79.18 g (86%) was achieved. A portion of this material was purified by sublimation (95 °C, 0.75 torr) for characterization, mp 146-147 °C (lit., 137-139 °C; lit., 155 °C; lit., 148 °C); δH (300 MHz; d6-DMSO) 4.50 (7 H, br, C(2)H); 8.71 (2 H, s, C(1)H, C(3)H); δC (300 MHz; d6-DMSO) 104.1 (C2), 176.4 (C1, C3); m/z (EI) 149.9317 (M+; C12H13BrNa2O2 requires 194.9034) 197(64%), 195(100), 159(38), 152(94), 174.2 (C4); 113.4 (C4a), 138.0 (C5), 154.6 (C7), 159.8 (C8a), 165.0 (C2), 174.2 (C4); m/z (ESI) 240.9725 (M+H+). C15H14BrN4O4 requires 347.0120) 349(98%), 347(98%), 345(98), and 303(32). Spectral data are identical to those reported for this compound in the literature.7

2-Pivaloylamino-6-bromo-4(3H)-pyridole (2-Pivaloyl-6-bromo-5-deazapterin) (S5).6 Bromo-5-deazapterin (S4) (5.00 g, 20.7 mmol) was sealed under Ar in a round bottom flask fitted with a reflux condenser. Pyridine (100 mL, 1.24 mol) and pivalyl chloride (7.65 mL, 62.1 mmol) were added with stirring. The reaction was heated at reflux temperature for 4 d. The reaction was then concentrated at 10 mm Hg and 45 °C until all visible solvent had been removed. The residue was dissolved in 125 mL anhydrous EtOH and heated at reflux temperature for 45 min to quench unreacted pivaloyl chloride before again being concentrated as above. The residue was diluted with CH2Cl2 and the solvent was allowed to evaporate overnight. Purification was achieved by plug filtration (silica, MeOH/CH2Cl2, 3:97), followed by flash chromatography (silica, MeOH/CH2Cl2, 1:99). Total yield 4.32 g (46%); δH (300 MHz; CDCl3; Me2Si) 1.34 (9 H, s, C(C3H3)); 8.13 (1 H, d, J = 2.6 Hz, C(5)H); 8.32 (1 H, d, J = 2.6 Hz, C(7)H); 12.17 (1 H, br s, C(2NH)). m/z (ESI) 347.0125 (M+Na+). C15H14BrN4O4 requires 347.0120 (M+Na+). C15H13BrN4O4 requires 349.0128 (349%, 347, 345, and 303(32). Spectral data are identical to those reported for this compound in the literature.6

Methyl 4-vinylbenzoate (S7).4 4-Vinylbenzoic acid (S6) (2.00 g, 13.5 mmol) was dissolved in MeOH (200 mL) under Ar. Concentrated H2SO4 (ca. 1.6 mL) was added dropwise, using a dropping funnel, with stirring. After the addition was complete, the reaction was heated at reflux temperature for 9 h while being monitored by TLC (EtOAc). The reaction was cooled and diluted with 200 mL CHCl3. This was washed with 200 mL saturated NaHCO3. The aqueous layer was then extracted with 200 mL CHCl3 and the combined organic phases were washed with 400 mL dH2O followed by drying with anhydrous MgSO4. The solution was filtered and concentrated in vacuo. The resulting yellow oil was purified by flash chromatography (silica, CH2Cl2). After concentrating and drying under high vacuum, white needles were obtained. Total yield 1.89 g (86%); mp 29-30 °C; δH (300 MHz; CDCl3; Me2Si) 3.84 (3 H, s, C(1)COOCH3); 5.31 (1 H, d, J = 10.9 Hz, C(4)CH=CH2); 5.80 (1 H, d, J = 17.6 Hz, C(4)CH=CH2); 6.67 (1 H, dd, J = 10.9, 17.6 Hz, C(4)CH=CH2); 7.37 (2 H, d, J = 8.3 Hz, C(3)H and C(5)H); 7.94 (2 H, d, J = 8.3 Hz, C(2)H and C(6)H). δC...
(300 MHz; CDCl3; Me6Si) 51.6 (C(1)COOCH3), 116.1 (C(4)CH=CH2), 125.8 (C3 and C5), 129.0 (C1), 129.6 (C2 and C6), 135.7 (C(4)CH=CH2), 141.6 (C4), 166.3 (C(1)COOCH3). Spectral data are identical to those reported for this compound in the literature.2

Methyl 4-(2-pivaloylamino-4(3H)-oxopyrrolo[2,3-d]pyrimidin-6-yl)-benzoate (Methyl 2-pivaloyl-5,10-dideaza-9,10-dehydro-pteroate) (S8).2 Methyl 4-vinylbenzoate (S7) (2.155 g, 13.28 mmol) and 6-bromo-2-pivaloyl-5-deazapterin (S5) (3.42 g, 13.28 mmol) were dissolved in CH2CN (150 mL) with stirring. Removal of O2 from the solution was effected by sparging with Ar for 15 min. Pd(OAc)2 (149 mg, 0.664 mmol), tri-o-tolyphosphine (404 mg, 1.328 mmol), and Et3N (11.1 mL, 79.68 mmol) were then added and a condenser was fitted to the vessel. An Ar atmosphere was established and maintained with the use of an Ar-filled balloon. The reaction was heated at reflux temperature for 30 h, after which time it was allowed to cool to ambient temperature overnight. The precipitate was collected by centrifugation (20 min, 4°C, 12,000 × g). The supernatant was decanted and 200 mL of fresh CH2CN was used to resuspend the pellet. Centrifugation and decantation was repeated as above. The pellet was then suspended in CH2CN and transferred to a round bottom flask. Concentration and drying yielded 4.63 g of crude product. The supernatants from the above washes were combined and concentrated, in vacuo, to dryness. The residue was suspended in 40 mL CH2CN and transferred to two 30 mL glass centrifuge tubes. These were then centrifuged (30 min, 4°C, 12,000 × g). The supernatant was removed and the pellets were suspended and combined with 20 mL CH2CN. Centrifugation and decantation was repeated as above. This was then repeated with another 20 mL CH2CN. After centrifuging and decanting, the resulting small pellet was combined with the large pellet obtained previously. Drying yielded a total pellet mass of 4.98 g. Purification was achieved by flash chromatography (silica, MeOH/CH2Cl2 5:95). All of the compound-containing fractions were concentrated in vacuo and dried under high vacuum. When S8 was excited by long wave UV light, a visible fluorescence was noted. Total yield 4.94 g (92%); δmax (0.1 M NaOD in D2O)/nm 242 and 279; (0.1 M NaOH)/nm 272; δm/z 300 MHz; δd/DMSO) 1.29 (9 H, s, C(9)H3). 3.87 (3 H, s, COOC(3). 7.61 (2 H, m, C(9)H=CH(10)H). 7.79 (2 H, J = 8.5 Hz, C(12)H and C(16)H). 7.99 (2 H, J = 8.5 Hz, C(13)H and C(15)H). 8.65 (1 H, J = 2.3 Hz, C(5)H). 9.16 (1 H, J = 2.3 Hz, C(7)H). 11.47 (1 H, br s, C(2)NH). 12.33 (1 H, br s, C(15)H); δd 500 MHz; CDCl3; Me6Si 25.4 (C5), 27.1 (C7), 31.4 (C9), 34.7 (C10), 45.6 (C7), 47.1 (C12), 52.1 (C8a), 73.2 (C14), 75.2 (C2), 77.1 (C3), 77.9 (C4), 78.3 (C13 and C15), 82.6 (C1), 87.7 (C4a), 128.0 (C3 and C5), 129.5 (C12 and C16), 134.0 (C14), 146.6 (C2), 147.0 (C11), 161.0 (C8a), 161.6 (C2), 172.0 (COOC(3)); RP-HPLC (Method 1), tR = 13.2 min, 97.6% purity. Chiral-HPLC, tR = 10.5, 14.8 min, 98.5% purity. Spectral data are identical to those reported for this compound in the literature.2

Methyl 2-pivaloyl-5,10-dideaza-9,10-dehydro-pterioate (6RS)-5,10-Dideaza-5,6,7,8-tetrahydropteroic acid (6RS-8).2 Methyl 2-pivaloyl-5,10-dideaza-6,7,8-tetrahydropteroic acid (6RS-7) (2.83 g, 6.86 mmol) was suspended in 68.6 mL of 1 M NaOH. A reflux condenser was fitted to the flask and the reaction was heated at reflux temperature for 30 min after the starting material had completely dissolved. The flask was then cooled to ambient temperature, and the solution was filtered through a paper filter, which was then washed with water. The pH of the filtrate was adjusted to 1 with 74 mL of 1 M HCl using pH 0-12 pH paper while stirring. The suspension was lyophilized to a small volume and resuspended in water for centrifugation. The suspension was transferred to four 30 mL glass centrifuge tubes which were then centrifuged (15 min, 4°C, 12,000 × g) to obtain the desired product as a pellet. The pellets were then washed with dH2O (2 × 70 ml), CH2CN (2 × 70 ml), and EtOAc (1 × 70 mL). The product was dried under high vacuum in the presence of P2O5. Product was determined to be 100% pure by RP-HPLC (Method 1). Total yield 2.195 g (91%); δmax (0.1 M HCl)/nm 242 and 279; (0.1 M NaOH)/nm 272; δm/z 300 MHz; δd/DMSO) 1.61 (3 H, m, C(6)H and C(10)H). 1.88 (1 H, m, C(5)H). 2.55 (1 H, m, C(9)H, partially obscured by residual DMSO peak), 2.72 (2 H, m, C(5)H and C(9)H). 2.85 (1 H, m, C(7)H). 3.29 (1 H, br d, J = 11.0 Hz, C(7)H2), 6.95 (2.3 H, br s, NH), 7.35 (2 H, d, J = 8.1 Hz, C(12)H and C(16)H). 10.90 (1 H, br s, C(15)H); δd 500 MHz; 0.1 M NaOD in D2O) 1.64 (2 H, m, C(10)H). 1.78 (1 H, m, C(6)H). 2.01 (1 H, dd, J = 9.1, 15.5 Hz, C(5)H). 2.57 (1 H, m, C(9)H). 2.76 (2 H, m, C(5)H and C(9)H). 2.88 (1 H, m, C(7)H). 3.27 (1 H, m, C(7)H). 7.34 (2 H, d, J = 7.6 Hz, C(12)H and C(16)H). 7.78 (2 H, d, J = 7.6 Hz, C(13)H and C(15)H); δd 500 MHz; 0.1 NaOD in D2O) 26.3(C5), 31.4(C6), 32.9(C9), 34.8(C10), 45.7(C7), 87.5(C4a), 128.7(C12 and C16), 129.5(C3 and C15), 134.0(C14), 147.0(C11), 161.0(C8a), 161.6(C2), 174.2(C4), 176.0(C17); m/z (EI+) 314.1381 (C19H14N2O4 requires 314.1379), 315.2(24%), 314.2(100), 179.1(23), 166.1(100), 165.1(90), 164.1(38), 163.1(41), 151.1(68), 149.1(26), 135.1(30), 107.1(21), 91.1(30), 77.1(25), 51.2(25), 40.0(33), 40.0(29), 40.0(29), 39.0(20), 36.0(44); RP-HPLC (Method 1), tR = 12.8 min. Spectral data are identical to those reported for this compound in the literature.2

Methyl 2-pivaloyl-5,10-dideaza-6,7,8-tetrahydropteroic acid (6RS-8).5 Methyl 2-pivaloyl-5,10-dideaza-6,7,8-tetrahydropteroic acid (6RS-9) (50 mg, 0.14 mmol) was added under Ar to a dry flask containing a stir bar. The flask was then sealed with a septum under an atmosphere of Ar (balloon) before the addition of 1 mL anhydrous DMF. The solution was cooled in a 20°C water bath before the addition of Et3N (112 µL, 0.8 mmol). After 15 min, cold ethyl chloroformate (15 µL, 0.154 mmol) was added. After 45 min, NaH (11 mg, 0.17 mmol) was then added. The reaction was allowed to stir for 1 h at ambient temperature. The reaction completion was verified by RP-HPLC, Method 1.
reaction solution was diluted with 6 mL of 0.1 M HCl for precipitation. The precipitate was collected by centrifugation (15 min, 4 °C, 12,000 g). The supernatant was removed and the pellet was washed with: 1 × 10 mL ddH2O; 1 × 5 mL Et2O. The pellet was allowed to air dry for 15 min before drying under high vacuum in the presence of P2O5 for several hours. Total mass recovery, 51 mg, a mixture of 6RS-9 (89.6%) and 6RS-8 based on RP-HPLC (Method 1); δH (300 MHz; d6-DMSO) 1.59 (3 H, m, C(6)H and C(10)H 2), 1.86 (1 H, m, C(5)H2), 2.12 (2 H, d, J = 8.7, 15.3 Hz, C(3)H 2, C(5)H 2), 2.52 (1 H, m, C(7)H2), 2.98 (2 H, d, J = 6.6 Hz, C(10)H2), 2.82 (2 H, d, J = 8.1 Hz, C(7)H2), 6.53 (2-3 H, br s, NH), 7.42 (2 H, d, J = 6.6 Hz, C(12)H and C(16)H), 7.90 (2 H, d, J = 8.1 Hz, C(13)H and C(15)H), 10.29 (1 H, br s, N(3)H); RP-HPLC (Method 1), tR = 30.4 min; Ion-pair HPLC, tR = 30.0 min. This material was used in subsequent coupling reactions without further purification.

(6RS)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid, DDAH4PteGlu3. A similar numbering system was used in the NMR assignments of all of the shorter chain-length γ-glutamyl peptides characterized above.

L-Glutamate-γ-L-glutamate (γ-Glu2) – Solution phase synthesis. N-Cbz-L-glutamate-γ-L-glutamate (0.314 g, 0.76 mmol) was dissolved in 10 mL ddH2O before the addition of 10% Pd/C (85 mg, 0.08 mmol). The solution was then hydrogenated at 40 psi H2 for 14 h. The reaction was filtered through Celite with the aid of ddH2O. The filtrate was further purified through size-exclusion chromatography but proved unsuccessful. The presumed trimmonium salt of the dipeptide was recovered by lyophilization. Total yield 190 mg. A portion of this material, 26.0 mg, was then purified by semi-preparative RP-HPLC (Method 15), tR = 10.0 min to obtain the TFA salt of the dipeptide after lyophilization. Total yield 24.8 mg (80%), δδ (500 MHz; 1% v/v TFA in D2O) 1.86 (1 H, m, C(8')H2), 2.07 (3 H, m, C(3')H2 and C(9')H2), 2.34 (2 H, m, C(9')H2), 2.40 (2 H, m, C(4')H2), 3.95 (1 H, dd, J = 4.1, 6.1 Hz, C(2')H), 4.26 (1 H, t, J = 4.3 Hz, C(7')H); δC (500 MHz; 1% v/v TFA in D2O) 25.7(C(8')), 25.9(C(3')), 30.3(C(9)'), 31.1(C(4)'), 52.4(C(7)'), 52.5(C(2)'), 171.7(C=O), 174.6(C=O), 175.3(C=O), 177.4(C=O); m/z (ESI+) 277.1034. (C10H17N2O7 requires 277.1036).

References

Scheme S2 Numbering system used for the NMR assignments of poly-γ-Glu6. A similar numbering system was used in the NMR assignments of all of the dipeptide after lyophilization of the dipeptide after lyophilization.
**Figure S1** A typical chromatogram of the preparative chiral HPLC method used for the separation of the isomers of 6RS-7. Eluant A – hexane. Eluant B – EtOH. Column – Chiralcel OJ, 2 cm x 25 cm. Isocratic at 70% B. Flow rate – 30 mL/min. Run time – 22 min. Sample concentration – 1.25 mg/mL in 25% A: 75% B. Monitored at 240 nm.

**Figure S2** A typical chromatogram of the analytical chiral HPLC method used for the resolution of 6RS-7 (Panel A) into its individual isomers, (6R)- (Panel B) and (6S)- (Panel C). Eluant A - ddH₂O. Eluant B – CH₃CN. Column – Chiral Technologies (DAICEL Chemical Industries, LTD) Chiralcel OJ-R, 4.6 x 150 mm. Isocratic at 40% B. Flow rate – 0.5 mL/min. Detection at 280 nm.