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Synthesis of γ-[^{15}N]-L-Glutamyl Derivatives of 5,10-Dideazatetrahydrofolate

Ronald A. Forsch and Andre Rosowsky*

Dana-Farber Cancer Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

SUMMARY

A synthesis of the mono-, di-, and tri[^{15}N]glutamate forms of the potent de novo purine synthesis inhibitor and anticancer agent (6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydrofolate (6*R*,6*S*-DDATHF) from (6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydropteroic acid is described. These isotopically labelled compounds are potentially useful as ^{15}N nmr probes of the interaction of DDATHF and its polyglutamates with three key enzymes of one-carbon metabolism, glycinamide ribonucleotide formyltransferase, (GARFT), aminoimidazolecarboxamide formyltransferase (AICARFT), and folylpolyglutamate synthetase (FPGS).

KEY Words: 5,10-dideazatetrahydrofolic acid, γ-oligo[^{15}N]glutamic acid derivatives

INTRODUCTION

Studies of the three-dimensional interaction of ^{15}N-labeled pharmaceuticals with biological macromolecules in solution can been obtained conveniently by ^{15}N nmr spectrometry (1,2). Use of

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this approach has been described for such diverse examples as the binding of N-hydroxybenzene-sulfonamides to carbonic anhydrase (3,4), of polyamines to tRNA (5), and of oligonucleotides (6,7) and peptides (8,9) to proteins. Interesting uses of ^{15}N nmr have also been made in structural studies of the enzyme dihydrofolate reductase (DHFR). Although most of the well-known studies done on this enzyme with the help of this technique have involved isotopic labelling of the backbone nitrogen atoms of the protein itself (10-14), an early application of ^{15}N nmr to characterize the binding of a ligand to the active site of the enzyme was made with a complex of the DHFR from *Lactobacillus casei* with the small-molecule inhibitor trimethoprim (15). In addition, studies of this type have been performed on complexes of isotopically labelled folic acid with *Lactobacillus casei* DHFR (16); of trimethoprim (17), folic acid (18), and methotrexate (18) with *Escherichia coli* DHFR; of folic acid and aminopterin mono- and pentaglutamates with bovine DHFR (19); and of folic and 7,8-dihydrofolic acid with human recombinant DHFR (20). The isotopic label in trimethoprim was on N1, N3, and the 2-amino group (15,17); in methotrexate, on N5 (18,21); and in folic and 7,8-dihydrofolic acid, on N3 and the 2-amino group (18,19). No studies have been reported on complexes in which both the inhibitor and the DHFR are isotopically labelled with ^{15}N.

Analyses of the three-dimensional interaction of substrates and inhibitors with enzymes of the folate pathway other than DHFR by ^{15}N nmr spectrometry have not been reported. Moreover, as noted above, introduction of ^{15}N into DHFR substrates and small-molecule inhibitors has been limited to the heterocyclic moiety. Anticipating that structural studies involving enzymes other than DHFR and ^{15}N labelling of parts of the substrate or inhibitor other than the heterocyclic moiety could be of interest, we synthesized the isotopically labelled compound (6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydro-pteroyl-L-[^{15}N]glutamate ([^{15}N]DDATHF, 1). Several synthetic routes to racemic DDATHF have been reported (23), as well as separation of the individual 6*R* and 6*S* diastereomers as (+)-camphor-sulfonate salts (24). An asymmetric synthesis has also been developed for the 6*R* isomer (25), which has been evaluated clinically under the name lometrexol (26). A chemical synthesis of (6*R*,6S)-DDATHF polyglutamates from (6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydropteroic acid (27) and an enzymatic synthesis of (6*R*)-DDATHF polyglutamates from (6*R*)-5,10-dideaza-5,6,7,8-tetrahydro-pteroic acid (28) have likewise been reported.

As shown in a series of papers (23,27-29), intracellular metabolism of DDATHF (and lometrexol) to non-effluxing γ-polyglutamates by the enzyme folylpolyglutamate synthetase (FPGS) plays a key role in the pharmacodynamics and pharmacokinetics of this drug. In particular, DDATHF

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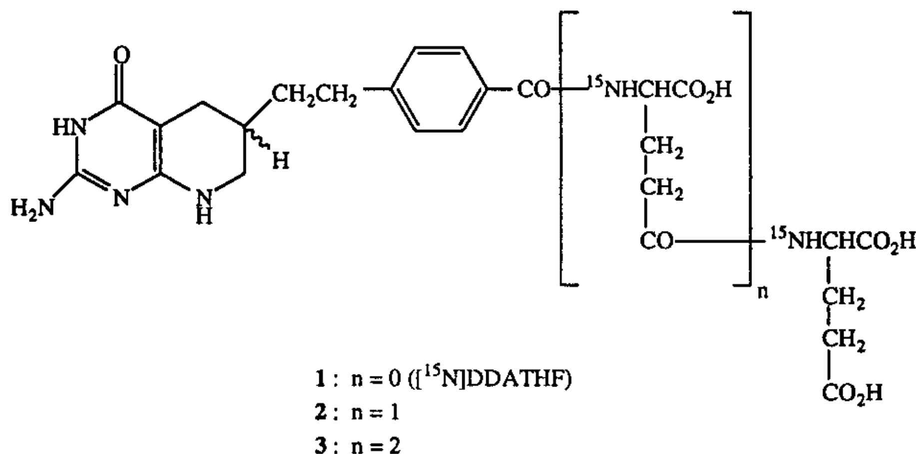
polyglutamation has been found to enhance binding to GARFT and give rise to more prolonged cellular retention than is the case with the parent monoglutamate. Interestingly, the 6*R* and 6S isomers are almost equiactive as inhibitors of de novo purine synthesis in cultured cells and bind to GARFT with K_i values that differ by less than twofold, suggesting that there is probably little difference in the spatial orientation of the two diastereomers within the active site of the enzyme. At high concentrations, DDATHF polyglutamates also bind tightly to another purine biosynthetic enzyme, aminoimidazolecarboxamide ribonucleotide formyltransferase (AICARFT). Thus, in addition to (6*R*,6*S*)-[^{15}N]-DDATHF (**1**) itself we prepared the ^{15}N-labelled di- and triglutamates **2** and **3** as potential ^{15}N nmr probes of the interaction of these ligands with the active site of FPGS, GARFT, and AICARFT. The chemical synthesis of **1-3** from (6*R*,6S)-5,10-dideaza-5,6,7,8-tetrahydropteroic acid (**4**) according to the reaction sequences depicted in Schemes I-III is the subject of this paper (30).



RESULTS AND DISCUSSION

As shown in Schemes I and II, condensation of dimethyl L-[^{15}N]glutamate (**5**) with (6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydropteroic acid (**4**), after reaction with 2-chloro-4,6-dimethoxy-*s*-triazine according to Barnett and coworkers (24), afforded the dimethyl ester **6** in 83% yield. Saponification of the ester with barium hydroxide in 50% aqueous ethanol then gave **1** in 68% yield. Ester **6** was purified to TLC homogeneity on a silica gel column, whereas the final product was purified by preparative HPLC on C_{18} silica gel with 7% acetonitrile in 0.05 *M* ammonium acetate, pH 6.9. Microchemical analysis of the lyophilized final product showed it to be a hydrated ammonium salt.

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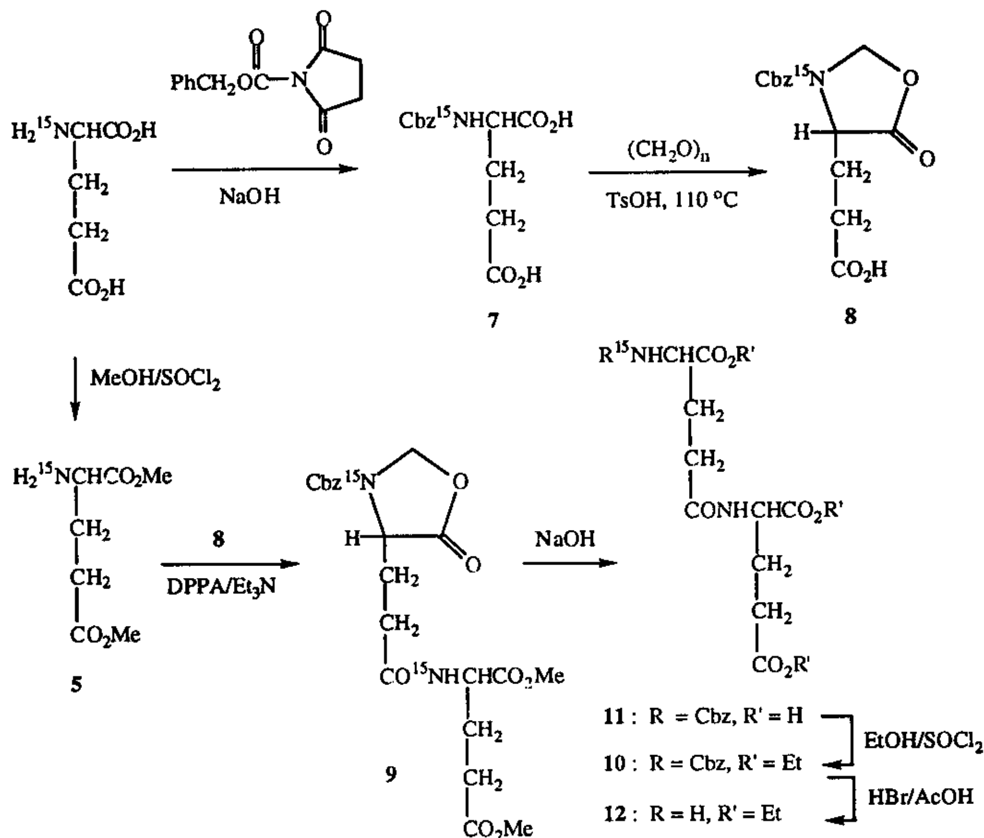
Scheme I



Treatment of [^{15}N]glutamic acid with N-(benzyloxycarbonyloxy)succinimide, a convenient alternative to the acid chloride, afforded the ^{15}N-benzyloxycarbonyl (Cbz) derivative **7** (Scheme I), which was converted directly to ^{15}N-Cbz-5-oxo-4-oxazolidinepropanoic acid (**8**) with paraformaldehyde and p-toluenesulfonic acid as described by Scholtz and Bartlett (31). The combined two-step yield of **8** was 84%. Condensation of **8** and **5** in the presence of diphenylphosphoryl azide and triethylamine afforded oxazolidinone **9**, which on saponification yielded the ^{15}N-Cbz triacid **10**. Re-esterification of **10** with ethanolic thionyl chloride followed by removal of the Cbz group with hydrogen bromide in glacial acetic acid afforded **11** and **12**·HBr, respectively (Scheme II). The combined four-step yield of **12**·HBr from **8** was 77%. Condensation of **12**·HBrwith **4**·HCl in the presence of 2-chloro-4,6-dimethoxy-*s*-triazine and the required amount of N-methylmorpholine afforded the coupled product **16**, which on saponification with sodium hydroxide in 50% aqueous

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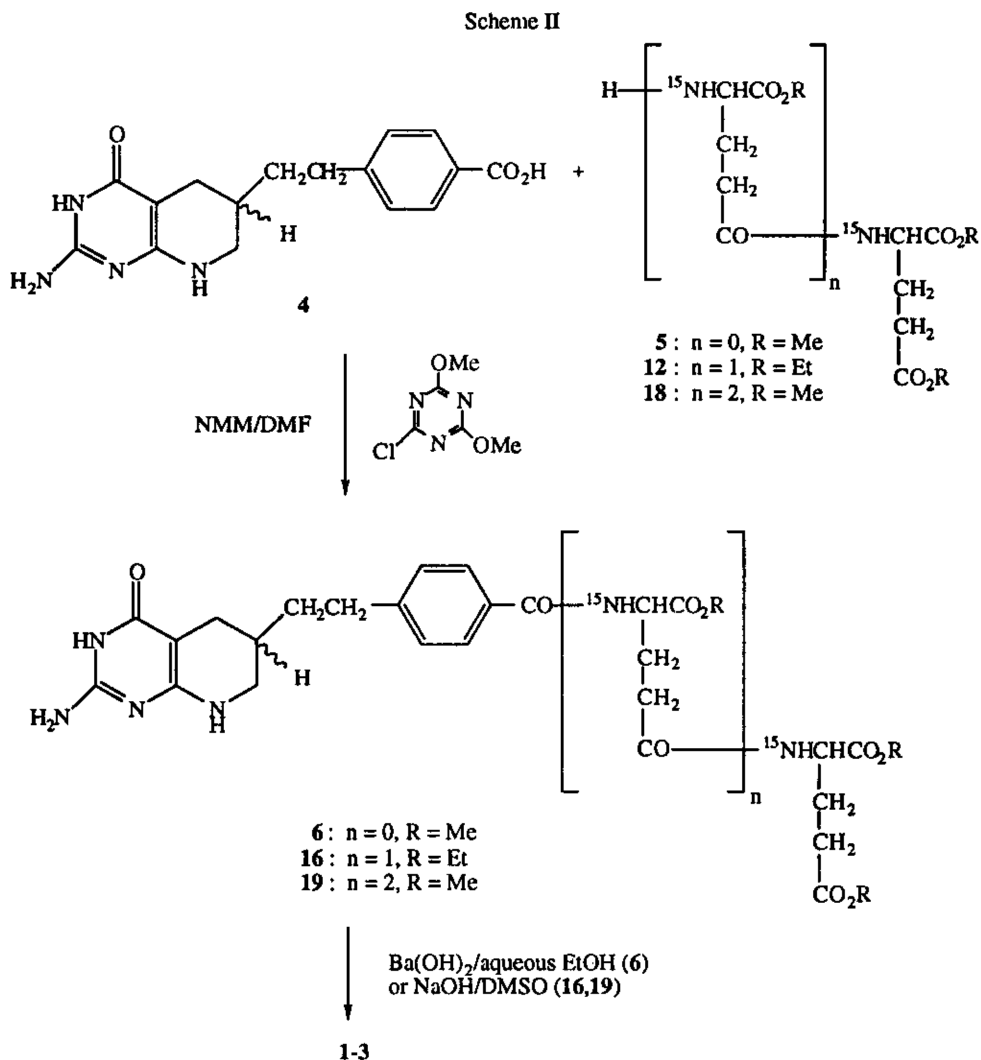

dimethyl-sulfoxide was converted to the γ-L-[^{15}N]glutamyl-L-[^{15}N]glutamyl derivative **2** in an overall two-step yield of 31% (Scheme II). Ethanol rather than methanol was used for the esterification of **10** because of passing concern about the possibility of oligomer formation during the ensuing coupling reaction of **12**·HBr in the presence N-methylmorpholine. In the event, however, this was not necessary, as we later found oligomerization not to be a problem (cf. below for the coupling reaction of the trimethyl ester **18**).

As with the lower homologs **6** and **1**, silica gel chromatography was used to purify **16** and preparative reversed-phase HPLC was used to purify **2**, in this case on a polymer-based column

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instead of a C_{18} silica gel column. Although we believe that polymer-based columns can decrease the risk of introducing inorganic silica into the sample, both types have been used satisfactorily in this work. It should be noted that even though intermediates **7-9** and even the heterocyclic coupling product **6** were all oils or gums, the purity of each compound was checked by TLC before taking it to the next step. Routine ^1H nmr spectra were also obtained, and generally featured the expected chemical shifts and peak areas. For example, the spectrum of **9** showed a complex multiplet at δ 1.8-2.9 for the two sets of CH_2CH_2 groups, a closely spaced pair of singlets at δ 3.65 and δ 3.70 for the O-methyl groups, a multiplet at δ 5.12 for the α-methinyl proton, and singlets at δ 5.18 and δ 7.38 for the CH_2 protons of the Cbz group and for the phenyl group. Structural changes such as the replacement of a methyl ester by an ethyl ester (e.g., conversion of **9** to **11**) or the removal of a Cbz group (e.g., conversion of **11** to **12**) were likewise routinely confirmed.

As depicted in Scheme III, treatment of oxazolidinone **8** with methanolic sodium methoxide led to **13** after crystallization of the dicyclohexylammonium salt as described in the literature (32). Condensation of **13** and **5** in the presence of diphenylphosphoryl azide and triethylamine, followed by removal of the Cbz group from **14** by hydrogenation in the presence of 5% palladium-on-carbon and a small amount of hydrochloric acid (generated in situ from acetyl chloride by reaction with the methanol solvent), afforded **15**·HCl with a combined two-step yield of 86%. Another diphenylphosphoryl azide coupling reaction, this time between **13** and **15**·HCl, afforded **17** in 86% yield, and this was followed again by catalytic hydrogenation in the presence of hydrochloric acid to obtain tripeptide **18**·HCl with an overall yield of 77% starting from **8**. All the intermediates formed in these steps were thick oils which stubbornly retained organic solvents and were never able to be crystallized. However routine TLC analysis and ^1H nmr spectra were used to be sure that each intermediate had the correct structure and was pure enough for the next step. Not surprisingly, the ^1H nmr spectra of these compounds showed considerable peak broadening consistent with the presence of similar but not identical $(CH_2)_2$, α-CH, and methyl ester groups. However by simply comparing the total area of the envelope corresponding to the $(CH_2)_2$ protons (δ 1.8-2.6) to that of the phenyl signal (δ 7.3) we could show for example that **14** was a dipeptide and **17** was tripeptide. In similar fashion, by comparing the total integrated area for the O-methyl signals to that of the envelope corresponding to the CH_2 protons of the Cbz group (δ 5.1), we could show that **14** was a triester and **17** was a tetraester. In addition, the peak ratio for the O-methyl signals of **14** and **17** were 2:1 and 3:1 respectively.

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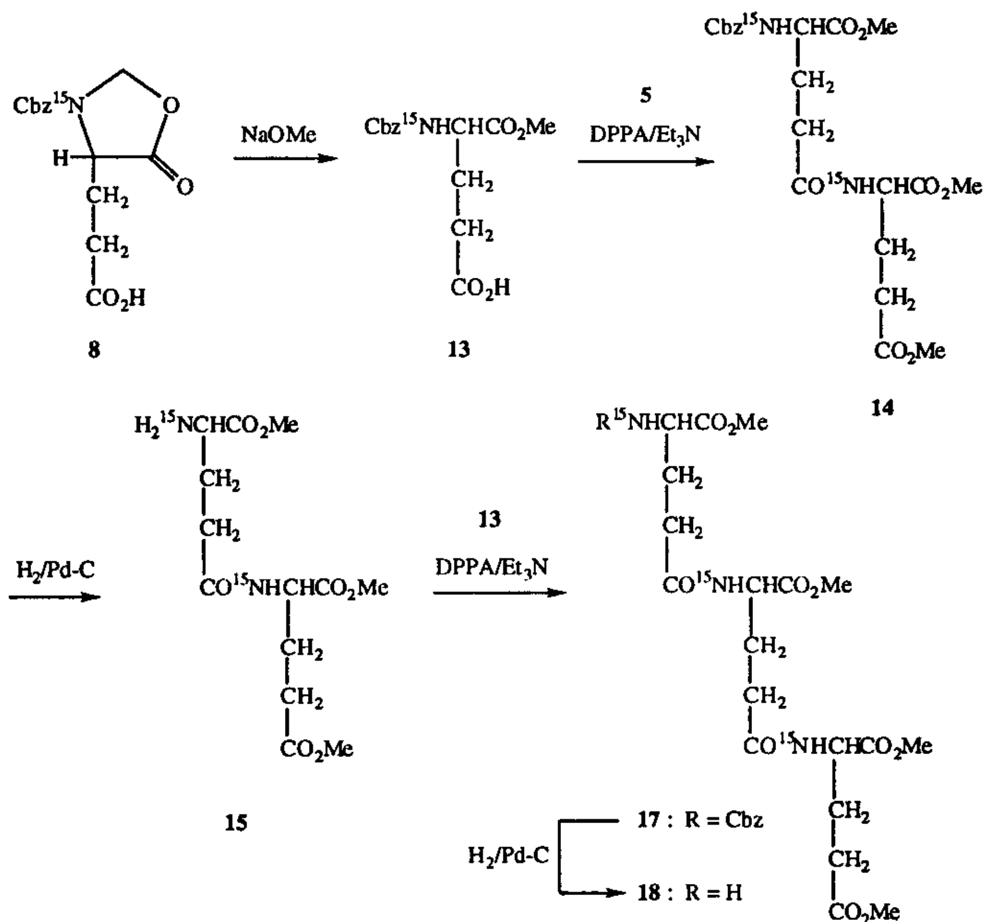
Scheme III



As shown in Scheme II, condensation of **18**·HCl and **4**·HCl with the aid of 2-chloro-4,6-dimethoxy-*s*-triazine and N-methylmorpholine afforded **19**, which on saponification with sodium hydroxide in 50% aqueous dimethylsulfoxide yielded the γ-L-[^{15}N]-glutamyl-γ-L-[^{15}N]glutamyl derivative **3**. After purification by DEAE-cellulose chromatography with ammonium bicarbonate buffers of different strength, followed by HPLC on C_{18} silica gel with 5% acetonitrile in 0.05 *M* ammonium acetate, pH 6.9, the combined two-step yield of **3** from **4**·HCl was only 18%. Thus the yields in the coupling and saponification steps unfortunately decreased in the order **1** (56%) > **2** (31%) > **3** (18%). The reason for the decreased yields in these reactions as an inverse function of chain length was not investigated but may reflect a tendency of the di- and triglutamates to undergo amide migration from the γ- to the α-position, for which there would be a twofold greater statistical probability in **3** than in **2** (33).

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Analytical HPLC was performed with all three of the [^{15}N]glutamate derivatives to compare their elution time under identical conditions. Using a C_{18} silica gel column which was eluted with a 40 min gradient of 1.0 to 15% acetonitrile in 0.1 *M* ammonium acetate, pH 6.0, the elution time of the monoglutamate (**1**) was 34 min, that of the diglutamate (**2**) was 31 min, and that of the triglutamate (**3**) was 29 min. The observed decrease in retention time as the number of carboxyl groups increased from two to four was consistent with our expectations.

EXPERIMENTAL

L-[^{15}N]glutamic acid was purchased from Cambridge Isotope Laboratory, Cambridge, MA. The hydrochloride salt of (6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydropteroic acid (**4**·HCl) was obtained from Dr. Chuan Shih, Lilly Laboratories, Indianapolis, IN. The acid is conveniently prepared by heating (6*R*,6*S*)-DDATHF in 6 *N* HCl at 100 °C in a sealed tube for 4 h (24). 2-Chloro-4,6-dimethoxy-*s*-triazine was prepared from the trichloro compound as described (31), and had a melting point of 75-76 °C after recrystallization from heptane. All compounds showed ^{1}H nmr spectra consistent with the assigned structures. Diphenylphosphoryl azide (DPPA), N-(benzyloxycarbonyl-oxy)succinimide, and N-methylmorpholine (NMM) were purchased from Aldrich, Milwaukee, WI. Other chemicals and solvents were from Fisher, Boston, MA. TLC was performed on Eastman 13181 silica gel sheets, and spots were visualized under a 254 nm UV lamp or in an I_2 chamber. Analytical HPLC was on a Waters C_{18} radial compression cartridge (5 μm particle size, 5 x 100 mm). Preparative HPLC was on a Waters C_{18} radial compression cartridge (15 μm particle size, 25 x 100 mm) or a Shodex DS-2013 stainless steel column packed with styrene-divinylbenzene polymer beads (8 μm particle size, 8 x 50 mm). HPLC peaks were monitored at 225 nm. Microanalyses were performed by Quantitative Technologies, Inc., Whitehouse, NJ.

N-[(6*R*,6*S*)-5,10-Dideaza-5,6,7,8-tetrahydropteroyl]-L-[^{15}N]glutamic Acid (1)

Step 1. A stirred suspension of L-[^{15}N]glutamic acid (148 mg, 1 mmol) in MeOH (20 mL) was cooled in an ice bath and treated dropwise with $SOCl_2$ (1 mL) over 5 min. The bath was removed, and after being left to stand at room temperature overnight the solution was evaporated to dryness to obtain dimethyl L-[^{15}N]glutamate hydrochloride (**5**·HCl) as a gum which was taken up in dry DMF (2 mL), and cooled to 0 °C. Separately, a solution of **4**·HCl (175 mg, 0.5 mmol) in dry DMF (5 mL)

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containing NMM (110 μL, 101 mg, 1.0 mmol) was cooled to 0 °C and treated with recrystallized 2-chloro-4,6-dimethoxy-*s*-triazine (88 mg, 0.5 mmol). After 1.5 h at 0 °C, a 1.1 ml portion of the foregoing solution of **5**·HCl (assumed to contain 0.5 mmol) was added, along with a second portion of NMM (55 μL, 51 mg, 0.5 mmol). The rest of the DMF solution of **5**·HCl was set aside for another run. After another 1 h of being stirred at 0 °C the reaction mixture was left at room temperature overnight, then concentrated to dryness by rotary evaporation with the aid of a vacuum pump. The residue was partitioned between $CHCl_3$ and water, and the organic layer was evaporated. TLC (silica gel, 10:1 $CHCl_3$-MeOH) showed a major spot at R_f 0.5 with faint impurities at the origin and at R_f 0.2 and 0.7. Chromatography on a silica gel column (15 g, 2 x 16 cm) with 10:1 $CHCl_3$-MeOH as the eluent afforded **6** as white solid which gave a single spot on TLC and was therefore used directly for the next step (195 mg, 83%), mp 168-180 °C; R_f 0.5 (silica gel, 10:1 $CHCl_3$-MeOH).

Step 2. A solution of **6** (174 mg, 0.369 mmol) in 50% EtOH (80 mL) was treated with solid $Ba(OH)_2 \cdot 8H_2O$ (348 mg, 1.1 mmol) and stirred at room temperature for 22 h. A solution of NH_4HCO_3 (237 mg, 3.0 mmol) in water (5 mL) was then added and the mixture was stirred vigorously for 5 min. The insoluble $BaCO_3$ salt was filtered off and the filtrate was concentrated to small volume by rotary evaporation. The solution was purified by preparative HPLC on C_{18} silica gel 7% MeCN in 0.05 *M* NH_4OAc, pH 6.9, 2 mL/min). A test sample from the main peak had a retention time of 34 min when re-chromatographed on an analytical C_{18} silica gel column using a 40 min gradient of 1.0 to 15% acetonitrile in 0.1 *M* ammonium acetate, pH 6.0. Pooled eluates from the main peak were concentrated and lyophilized, the residue was taken up in distilled water containing a small amount of ammonia, and the solution was lyophilized again to obtain a white solid (126 mg, 68%). Microanalytical data were consistent with a hydrated ammonium salt. Anal. $C_{21}H_{25}(^{14}N)_4(^{15}N)O_6 \cdot NH_3 \cdot 3H_2O$: C, 48.93; H, 6.65; N, 16.49. Found: C, 48.69; H, 6.46; N, 16.88.

γ-[N-[N-[(6*R*,6*S*)-5,10-Dideaza-5,6,7,8-tetrahydropteroyl]-L-[^{15}N]glutamyl]]-L-[^{15}N]-glutamic acid (2)

Step 1. A solution of L-[^{15}N]glutamic acid (0.59 g, 4.0 mmol) in a mixture of dioxane (20 mL) and 0.4 *M* NaOH (20 mL) was treated with N-(benzyloxycarbonyloxy)succinimide (1.2 g, 4.8 mmol) at 90 °C for 1 h. The dioxane was evaporated under reduced pressure, and the product was partitioned between EtOAc and water. The aqueous layer was acidified with HCl and extracted with EtOAc.

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Evaporation of the organic layer yielded N-(benzyloxycarbonyl)-L-[^{15}N]glutamic acid (**7**) as a partially crystallized gum (1.1 g, 94%) which was used with no additional purification.

Step 2. A mixture of **7** (1.1 g, 3.8 mmol), paraformaldehyde (0.24 g, 8.0 mmol), and TsOH·H_2O (40 mg) in toluene (100 mL) was refluxed in a Dean-Stark apparatus for 30 min. The cooled mixture was diluted with EtOAc until it became clear, and then was washed with a solution of $NaHCO_3$ (16 mg) in a few mL of water. Evaporation of the organic layer afforded **8** as an oil (0.99 g, 89%) which was used with no additional purification.

Step 3. A solution of **8** (147 mg, 0.5 mmol), **5**·HCl (106 mg, 0.5 mmol), and DPPA (108 µL, 138 mg, 0.5 mmol) in dry DMF (5 mL) was cooled in an ice bath and treated with Et_3N (209 µL, 152 mg, 1.5 mmol). The reaction mixture was kept in the ice bath for 1 h and left at room temperature overnight, and the product was partitioned between EtOAc and water. The organic layer was washed with 0.2 *N* HCl, rinsed to neutrality with water, and evaporated under reduced pressure to obtain the oxazolidinone **9** as an oil (211 mg, 93%) which was used with no additional purification.

Step 4. A solution of **9** (211 mg, 0.467 mmol) in MeOH (10 mL) was treated with 1 *M* NaOH (3 mL) and left to stand at room temperature overnight. After removal of the MeOH by rotary evaporation, the aqueous mixture was acidified with 1 *N* HCl (5 mL) and extracted several times with EtOAc. The combined organic layers were washed with water and evaporated to dryness, affording the amino triacid **10** as a gum (180 mg, 94%) which was used with no additional purification.

Step 5. A stirred solution of **10** (180 mg, 0.437 mmol) in absolute EtOH (50 mL) was cooled to 0 °C and treated dropwise with $SOCl_2$ (2 mL) over 5 min. After being stirred at room temperature for 20 h the solution was evaporated to dryness under reduced pressure, and the residue was dried in vacuo at 60 °C to obtain the amino triester **11** as a thick oil (200 mg, 92%) which was used directly in the next step; R_f 0.4 (silica gel, $CHCl_3$).

Step 6. A solution of **11** (189 mg, 0.381 mmol) in glacial AcOH (1 mL) was treated with 30% HBr-AcOH (1 mL) and left to stand at room temperature for 1.5 h. Et_2O (100 mL) was added and the flask was placed in the freezer overnight. The liquid was poured off, leaving **12**·HBr as a brown gum (162 mg, 96%) which was used directly in the next step without neutralization.

Step 7. A solution of **4**·HCl (116 mg, 0.33 mmol) and NMM (72 µL, 67 mg, 0.66 mmol) in dry DMF (5 mL) was cooled in an ice bath and treated with 2-chloro-4,6-dimethoxy-*s*-triazine (58 mg, 0.33 mmol). After being stirred at 0 °C for 1 h, the mixture was treated with a solution of **12**·HBr (162 mg, 0.366 mmol) in DMF (2 mL) followed by a second portion of NMM (44 µL, 40 mg, 0.40 mmol).

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After being stirred at 0 °C for 1 h and left at room temperature overnight, the reaction mixture was evaporated under reduced pressure and the remaining crude product was stirred vigorously with water (10 mL). The aqueous wash was poured off and the product was applied onto a silica gel column (10 g, 1.5 x 18 cm) which was eluted with 10:1 $CHCl_3$-MeOH. Fractions containing the triester **16** according to TLC (R_f 0.5, silica gel, 10:1 $CHCl_3$-MeOH) were pooled, evaporated to dryness, and taken up in DMSO (2 mL). The solution was treated with 1 *M* NaOH (2 mL) for a few minutes, then diluted with water (8 mL), adjusted to pH 8 with 10% AcOH, and further diluted with water (8 mL). The solution was applied onto a preparative HPLC column (styrene-divinylbenzene polymer, 18% MeCN in 0.1 *M* NH_4OAc, pH 8.0, 3 mL/min). A test sample from the main peak had a retention time of 31 min when re-chromatographed on an analytical C_{18} silica gel column a 40 min with a gradient of 1.0 to 15% acetonitrile in 0.1 *M* ammonium acetate, pH 6.0. The residue after lyophilization of pooled fractions from the main peak was taken up in dilute ammonia and the solution was re-lyophilized. Further drying in vacuo at 60 °C afforded **2** as a hygroscopic white solid (73 mg, 31% combined yield from **4**·HCl). The microanalytical data were consistent with a hydrated sodium-ammonium salt. Anal. Calcd for $C_{26}H_{32}(^{14}N)_4(^{15}N)_2O_9 \cdot Na \cdot 0.5NH_3 \cdot 6H_2O$: C, 43.79; H, 6.29; N, 13.05. Found: C, 43.92; H, 5.92; N, 13.22.

γ-[N-[γ-[N-[N-[(6*R*,6*S*)-5,10-dideaza-5,6,7,8-tetrahydropteroyl]-L-[^{15}N]glutamyl]]-L-[^{15}N]glutamyl]-L-[^{15}N]glutamic acid (3)

Step 1. A solution of oxazolidinone **8** (940 mg, 3.20 mmol) in MeOH (20 mL) was treated with 7.8 mL of a 1.4 *M* solution of NaOMe in MeOH. The solution was kept at room temperature for 40 min and then neutralized with 1 *N* HCl (13 mL). The MeOH was evaporated and the residue was partitioned between EtOAc and 0.1 *N* HCl. The organic layer was evaporated under reduced pressure to obtain the monoacid **13** as an oil (870 mg, 92%). The oil was re-dissolved in EtOAc (20 mL), N,N-dicyclohexylamine (597 µL, 543 mg, 3.0 mmol) was added, and the solution was allowed to evaporate passively until crystals of the dicyclohexylammonium salt (**13**·DCHA) were formed (1.25 g, 90%); mp 166-168 °C (lit. mp 171-173 °C) (34).

Step 2. A solution of **13**·DCHA (382 mg, 0.8 mmol) in 0.2 *N* HCl was extracted with EtOAc and the organic layer was evaporated to dryness to obtain the free monoacid **13** as an oil, which was taken up in DMF (10 mL). A solution of **5** (164 mg, 0.77 mmol) in a minimal volume of DMF was added, and the mixture was cooled in an ice bath and treated with DPPA (220 mg, 0.8 mmol) followed by Et_3N

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(418 μL, 304 mg, 3.0 mmol). The stirred mixture was allowed to come to room temperature overnight and the solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc and the solution was washed with water followed by 1 N HCl, then water followed by 5% $NaHCO_3$, and finally water again. Evaporation to dryness followed by chromatography on silica gel (14 g, 1.5 x 20 cm) with 50:1 $CHCl_3$-MeOH as the eluent afforded triester **14** as a gum (257 mg, 71%); R_f 0.6 (silica gel, $CHCl_3$, detection with I_2).

Step 3. A solution of **14** (174 mg, 0.38 mmol) in MeOH (40 mL) to which acetyl chloride (0.1 mL) had been added to generate HCl in situ was shaken for 17 h with 5% Pd-C (50 mg) under H_2 (3 atm) in a Parr apparatus. Filtration of the catalyst through a Celite bed and evaporation of the filtrate under reduced pressure afforded crude **15**·HCl as a gum which was taken up directly in DMF (ca. 1 mL). Separately a solution of **13**·DCHA (181 mg, 0.38 mmol) was partitioned between EtOAc and 1 N HCl, and the EtOAc layer was evaporated to obtain the free acid as a gum, which was combined directly with the solution of **15**·HCl using enough DMF to effect transfer and bring the final volume to 10 mL. The solution was cooled in an ice bath and treated sequentially with DPPA (105 mg, 0.38 mmol) and Et_3N (167 μL, 121 mg, 1.2 mmol). After being left to stand at room temperature overnight the solvent was evaporated under reduced pressure and the residue was partitioned between EtOAc and water. The organic layer was washed consecutively with 1 *N* HCl, water, 5% aqueous $NaHCO_3$, and water. Concentration to dryness on the rotary evaporator afforded **17** as a gum (86%) which was used directly for the next step; TLC: R_f 0.6 (silica gel, 20:1 $CHCl_3$-MeOH, detection with I_2).

Step 4. A solution of **17** (195 mg, 0.326 mmol) in MeOH (40 mL) to which acetyl chloride (0.1 mL) had been added to generate dry HCl in situ was shaken for 17 h with 5% Pd-C (50 mg) under H_2 (3 atm) in a Parr apparatus. Filtration of the catalyst through a Celite bed and evaporation of the solvent under reduced pressure yielded **18**·HCl as a glass (164 mg, 100%) which used with no additional purification; R_f 0.3 (silica gel, 20:1 $CHCl_3$-MeOH, detection with I_2).

Step 5. A solution of **4**·HCl (112 mg, 0.32 mmol) in dry DMF (5 mL) containing NMM (70 μL, 65 mg, 0.64 mmol) was cooled in an ice bath and treated with 2-chloro-4,6-trimethoxy-*s*-triazine (56 mg, 0.32 mmol). The solution was kept at 0 °C for 1 h, and to it was then added the DMF solution of **18**·HCl from the preceding step, followed by another portion of NMM (35 μL, 32 mg, 0.32 mmol). The reaction mixture was allowed to stand at room temperature for 3 days and concentrated to dryness by rotary evaporation with the aid of a vacuum pump. The residue was shaken vigorously with water (10 mL) and the solid, consisting of **19**, was filtered and dissolved in DMSO (1 mL) to which was

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then added 2 *M* NaOH (2 mL). After a few minutes the solution was diluted to 10 mL with distilled water, adjusted to pH 7 with 10% AcOH, and filtered. The filtrate was applied onto a DEAE-cellulose column (1.5 x 26 cm, HCO_3^- form) and the column was eluted with water to remove the DMSO, then with 0.1, 0.2, and 0.4 *M* NH_4HCO_3, and finally with 0.4 *M* NH_4HCO_3 adjusted to pH 10 with ammonia. Most of the UV-absorbing material was found in the 0.4 *M* NH_4HCO_3 eluate, with smaller amounts eluting in the other fractions. The largest UV-absorbing peak was collected and lyophilized to obtain a white solid (80 mg) which was still contained a small impurity according to analytical HPLC (C_{18} silica gel, 5% MeCN in 0.05 *M* NH_4OAc, pH 6.9, 2 mL/min). Preparative HPLC with the same eluent system removed the impurity and afforded **3** as a colorless solid (46 mg, 18% combined yield from **4**·HCl). A test sample from the main preparative HPLC peak had a retention time of 29 min when re-chromatographed on an analytical C_{18} column using a 40 min linear gradient of 1.0 to 15% acetonitrile in 0.1 *M* ammonium acetate, pH 6.0. The microanalytical data were consistent with a hydrated ammonium salt. Anal. Calcd. for $C_{31}H_{39}(^{14}N)_4(^{15}N)_3O_{12}\cdot 2.25NH_3\cdot 4H_2O$: C, 45.68; H, 6.65; N, 16.26. Found: C, 45.57; H, 6.21; N, 16.36.

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