Synthesis of L-2-(N-Pteroylamino)-3-(N-phosphonoacetyl)aminopropanoic Acid as an Analogue of the Putative Phosphorylated Intermediate in the γ -Glutamation of Folic Acid by Folylpolyglutamate Synthetase

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Summary

L-2-(N-Pteroyl)amino-3-(N-phosphonoacetyl)aminopropanoic acid was synthesized as an analogue of the putative γ -phosphorylated intermediate in the enzyme-catalyzed γ -glutamation of folic acid by folylpolyglutamate synthetase (FPGS). N-(Benzyloxycarbonyl)-L-aspartic acid was converted in four steps to methyl L-2-(N-benzyloxycarbonyl)amino-3-aminopropanoate, and the latter was allowed to react with p-nitrophenyl dimethoxyphosphonoacetate to obtain methyl L-2-(N-benzyloxycarbonylamino)-3-(N-dimethoxyphosphonoacetyl)aminopropanoate. After catalytic hydrogenation, the resulting amine was coupled to N¹⁰-formylpteronic acid via the mixed carboxylic-carbonic anhydride method, and the three ester groups were removed by sequential treatment with Me₃SiBr in DMF and NaOH in DMSO. When the last step was performed only with NaOH/DMSO, one of the phosphonate esters remained intact, giving L-2-(N-pteroyl)amino-3-(N-monomethoxyphosphonoacetyl)aminopropanoic acid. Also synthesized as a potential FPGS inhibitor was N ^{α} -(4-amino-4-deoxy-N¹⁰-methylpteroyl)-N ^{ϵ} -phosphonoacetyl-L-lysine. The ability of these phosphonoacetyl derivatives to inhibit catalytic addition of L-glutamic acid to folic acid proved to be very low, suggesting that replacement of the CH₂C(=O)OP(=O)(OH)₂ moiety by NHC(=O)CH₂P(=O)(OH)₂ may place the terminal phosphonyl group in an unfavorable spatial orientation for binding to the enzyme.

Key words : Folic acid, Folylpolyglutamate synthetase, γ -Phosphorylated intermediate analogue

Introduction

Consecutive addition of one or more L-glutamyl moieties to the γ -carboxyl group in natural folates and glutamate-containing antifolates plays a critical role in the cellular pharmacology of these physiologically important molecules

(reviewed in refs. 1 and 2). The reaction is catalyzed by the enzyme folylpolyglutamate synthetase (FPGS), whose requirement for normal cell growth was originally inferred from the observation that FPGS-deficient cells are auxotrophic for thymidine and a purine (3,4). More recently a required role for FPGS in normal cell growth was demonstrated by Priest and coworkers (5), using an antisense expression system to

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down-regulate FPGS activity in CCRF-CEM human leukemic cells. Commensurate decreases in total reduced folate pools, thymidylate synthesis, and growth rate were observed (5). The multiple negative charges in the side chain of folyl γ -polyglutamates retard the efflux of these molecules from cells, and also makes them better substrates than the parent monoglutamate for various folate-metabolizing enzymes, such as thymidylate synthase. Thus, γ -polyglutamation of natural folates serves a dual purpose, in helping to maintain adequate cofactor pools while ensuring that reduced folate cofactors are utilized with maximum catalytic efficiency. In the cellular pharmacology of a number of important antifolates, γ -polyglutamation serves a similar dual purpose except that tighter enzyme binding enhances inhibitor activity rather than substrate activity. The use of FPGS inhibitors as drugs was proposed more than 15 years ago (6). Since then, a number of potent substrate analogues have been tested, including for example compounds with a homocysteic acid (7a-c), 2-amino-4-phosphonobutanoic acid (7b, 7c, 8), ornithine (7c, 9a-f), or 2-amino-4-(1H-tetrazol-1-yl)butanoic acid (10) side chain. Other analogues with γ -substitution on the glutamate moiety (11a-c) or with fluorine substitution on the ornithine moiety (12) have also been studied. However none of the substrate analogues studied to date have shown enough preclinical activity to warrant clinical trial.

An elegant isotopic study (13) utilizing folic acid (**1**) as the substrate suggested that the first step along the FPGS reaction coordinate is the transient formation of the acylphosphate intermediate **2** (Fig. 1). This is followed by rapid phosphate displacement by the incoming glutamate moiety to give folyl- γ -L-glutamate (**3**). Further rounds of phosphate transfer from ATP and phosphate displacement by L-glutamate generates oligomers of different chain-length depending on the kinetics of each successive elongation step.

On the basis of this mechanism, a reasonable approach to the development of potent inhibitors of the FPGS reaction might be to design stable analogues of the acylphosphate intermediate **2**. A preliminary step in this direction was taken by us some years ago with the observation that the N-(phosphonoacetyl)-L-lysine analogue **4** (Scheme 1) could retard the γ -glutamation of folic acid by murine FPGS (6). However two structural features of this molecule were undesirable in terms of inhibitor design. In the first place, as a 2,4-

Figure 1.

diamino derivative, **4** would almost certainly be a potent inhibitor of dihydrofolate reductase (DHFR), so that any effect on cell growth would not be unequivocally attributable to FPGS inhibition alone. Secondly, the side chain in **4** was longer by two atoms than that of the putative acylphosphate intermediate **2**, an arrangement that we suspected would be less than optimal for binding to the enzyme active site. In the present paper we present details of our original synthesis of **4**, and also report the synthesis of L-2-(N-pteroyl)amino-3-(N-phosphonoacetyl)aminopropanoic acid (**5**) (Scheme 2). The latter is viewed as a closer analogue of **2**, in that (a) the heterocyclic moiety is a 2-amino-4-hydroxypteridine rather than a 2,4-diaminopteridine, and (b) the side chain is of the same length as that of **2**. Indeed, the only difference between **1** and **2** is that the $\text{CH}_2\text{P}(=\text{O})\text{O}$ moiety in the side chain is replaced by $\text{NHP}(=\text{O})\text{CH}_2$.

Chemistry

As indicated in Scheme 1, the synthesis of **4** was accomplished in a single step, albeit in modest yield, from phosphonoacetyl chloride and the previously described compound 4-amino-4-deoxy- N^{10} -methylpteroyl-L-lysine (**6**) (14a, b).

Scheme 1.

The reaction was carried out under Schotten-Baumann conditions, with the amine being added slowly to a solution of the acid chloride in aqueous dioxane containing excess NaOH. The product was partially desalted by passing it

through a Sephadex G10 size-exclusion column with water as the eluent, and was further purified by chromatography on a DEAE-cellulose column which was eluted successively with deionized water and 3% ammonium bicarbonate. The ammonium bicarbonate eluent was monitored by TLC, and fractions containing a single spot were combined and freeze-dried. Microchemical analysis showed **4** to be a partial ammonium salt, and because the final test sample was dried only by lyophilization (no heat) it also contained 4.5 molecules of water.

As outlined in Scheme 2, the synthesis of **5** began with N-(benzyloxycarbonyl)-L-aspartic acid (**7**). The procedure of Scholtz and Bartlett (15), with minor modifications, was used to convert **7** to the oxazolidinone **8** by reaction with paraformaldehyde and p-TsOH, and thence to the protected L-2,3-diaminopropanoic acid derivative **9** via Curtius rearrangement. Treatment of **9** with NaOMe gave the ester **10**, which on acidolysis with p-TsOH was converted to the tosylate salt **11**.

^a Reagents: (a) $(\text{CH}_2\text{O})_n/\text{TsOH}$ (b) DPPA/ $\text{Et}_3\text{N}/t\text{-BuOH}$; (c) NaOMe (d) TsOH (e) $4\text{-O}_2\text{NC}_6\text{H}_4\text{OC}(=\text{O})\text{CH}_2\text{P}(=\text{O})(\text{OMe})_2$ (**12**)/ Et_3N (f) $\text{H}_2/\text{Pd-C}$ (g) 10-Formylpteroid acid (**15**)/ $i\text{-BuOCOC}/\text{Et}_3\text{N}/\text{DMF}$ (h) $\text{Me}_3\text{SiBr}/\text{DMF}$ (i) NaOH/DMSO

Scheme 2.

11. Phosphonoacetylation of **11** was performed with 4-nitrophenyl dimethoxyphosphonoacetate (**12**), which we synthesized from 4-nitrophenol and dimethoxyphosphonoacetic acid in the presence of dicyclohexylcarbodiimide (DCC). The acid was readily obtained by selective hydrolysis of the carboxylic ester group in trimethylphosphonoacetate (**16**). Compound **12** had been made earlier from 4-nitrophenyl bromoacetate and trimethylphosphite via the Arbuzov reaction (**17**). Our method obviates the need to use toxic trimethylphosphite. Removal of the Cbz group by catalytic hydrogenation afforded the dimethoxyphosphonoacetylated intermediate **14**. To complete the synthesis, **14** was coupled to N¹⁰-formylpteroic acid (**15**) (**18**) via the mixed carboxylic-carbonic anhydride method (*i*-BuOCOCl/Et₃N) and the resulting product (**16**) was treated consecutively with Me₃SiBr to deprotect the phosphonate group and with NaOH to remove the ester and N¹⁰-formyl groups. As expected, **16** was insoluble in CHCl₃ and very poorly soluble in MeOH, and thus could not be purified by column chromatography on silica gel using these typical solvents. Fortunately, lack of solubility in nonpolar solvents did not preclude the use of Me₃SiBr, as this reaction proceeded quite well in DMF. Subsequent removal of the N¹⁰-formyl and ester groups with NaOH occurred rapidly in DMSO solution (5 min at 60°C). After the ester and amide protecting groups had been removed the product became water-soluble and could be purified for analysis by reversed-phase HPLC on C₁₈-silica gel with 4% MeCN in 0.05 M NH₄OAc as the eluent. The ¹H NMR spectrum of **5** in DMSO-*d*₆ solution showed the expected C₇ proton as a singlet at δ 8.62, the phenyl protons as a pair of doublets at δ 6.53 (3',5'-H) and 7.55 (2',6'-H) respectively, and the CH₂ group next to P as a doublet at δ 3.52, partially overlapping the β -CH₂ group. No signals attributable to OMe groups or the N¹⁰-formyl group were detected, showing that deprotection was complete. As in the case of **4**, the microanalytical data were consistent with a hydrated monoammonium salt.

The major product of the reaction of **16** with one equivalent of NaOH was the monomethoxyphosphinyl derivative **17**. As with **5**, purification of **17** was achieved by C₁₈ silica gel preparative HPLC, but with 5% rather 4% MeCN as the organic phase. The structural identity of **17** was established from its ¹H NMR spectrum, which showed a P-OMe doublet overlapping the β -CH₂ doublet, but was otherwise very similar to

that of **5** after allowing for solvent differences (D₂O versus DMSO-*d*₆). Microchemical analysis again showed **17** to be a hydrated ammonium salt. It may be noted that a minor HPLC peak which eluted more slowly than **17** was observed during its purification, which might have been **18**. However because we were concerned that it would have very poor aqueous solubility, this slower-moving product was not collected and further efforts to isolate and characterize **18** were not deemed worthwhile.

Interaction with FPGS

Experiments were performed to determine whether **4**, **5** and **17** inhibit FPGS-catalyzed addition of L-glutamic acid to folic acid under the assay conditions reported previously (**19**). The ability of these compounds to block the polyglutamation reaction was found to be very poor, with only slight inhibition being observed at 100 μ M. Since the reported *K_m* of folic acid as substrate is approximately 140 μ M (**20**), the *K_i*/*K_m* ratios for our N-phosphonoacetyl derivatives is obviously much less favorable than that of a number of other known FPGS inhibitors of either the pterin or 2,4-diaminopteridine type. For example the *K_i* values reported for N ^{α} -pteroyl-L-ornithine (**19**) and 4-amino-4-deoxypteroyl-L-ornithine (**20**) against murine FPGS are only 3.5 μ M (**21**) and 0.15 μ M (**9c**), respectively, corresponding to *K_i*/*K_m* ratios of ca. 40:1 and 900:1 relative to folic acid. Several other FPGS inhibitors synthesized in our laboratory have given *K_i* values in the nanomolar range, the best of them being N ^{α} -(5,8-dideazapteroyl)-L-ornithine (**21**) (*K_i*=0.35 μ M, *K_i*/*K_m* ca. 400), N ^{α} -(4-amino-4-deoxy-5,8-diazapteroyl)-L-ornithine (**22**) (*K_i*=0.072 μ M, *K_i*/*K_m* ca. 1900), and N ^{α} -(4-amino-4-deoxy-8-deazapteroyl)-L-ornithine (**23**) (*K_i*=0.018 μ M, *K_i*/*K_m* ca. 7800 (**9c**). In experiments utilizing FPGS from pig liver rather than mouse liver, two other ornithine-containing analogues, N ^{α} -(5,8-dideaza-5-chloropteroyl)-L-ornithine (**24**) and N ^{α} -(4-amino-4-deoxy-5-chloropteroyl)-L-ornithine (**25**), were found to be even more potent, with *K_i* values of 0.0083 μ M (*K_i*/*K_m* ca. 11,000) and 0.0017 μ M (*K_i*/*K_m* ca. 55,000) respectively (**9d**). Thus, despite its structural resemblance to γ -folyl phosphate (**2**), the putative activated intermediate in the FPGS reaction (Fig. 1), the affinity of **5** for the enzyme is clearly inferior to that of simpler ornithine analogues.

Figure 2.

It is of interest to note that an alternative approach to the development of mechanism-based inhibitors of FPGS was recently described by Tsukamoto and coworkers (22) in the form of compound **26**, which can be viewed as a bisubstrate analogue combining **1** and L-glutamate into a single molecule. Interestingly, **26** may be also viewed an analogue of **2** itself (i.e., a product analogue). The K_i of **26** as an inhibitor of the catalytic addition of L-glutamate to MTX in the presence of human FPGS was found to be 0.12 μM , whereas the corresponding ornithine analogue had a K_i of 3.2 μM (22). While **26** is not more potent than some of the other ornithine analogues discussed above, previous structure-activity correlation studies (9a-c) suggest that the potency of **26** against FPGS would probably be enhanced by replacing the N^{10} -methyl by hydrogen and by replacing N^5 and N^8 by carbon. On the other hand a disadvantage of **26** as an FPGS-targeted drug is that its affinity for DHFR is three orders of magnitude higher than its affinity for FPGS (22). As a result, any cell growth inhibition observed with this compound is most likely due to DHFR rather than FPGS inhibition. Nevertheless, the fact that **26** binds better than **5** to FPGS is of interest, in that it suggests that structures that resemble later intermediate species along the FPGS reaction coordinate may represent a more promising approach

to the design of effective mechanism-based inhibitors than structures based on the putative early intermediate **2**.

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Experimental Section

General

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer. Shoulders and very weak peaks are omitted. ^1H NMR spectra at 60 MHz were recorded on a Varian Model EM360L instrument, using Me_4Si as the internal reference for spectra in CDCl_3 and $\text{DMSO}-d_6$ and $\text{Me}_3\text{SiCD}_2\text{CD}_2\text{COONa}$ as the internal reference for spectra in D_2O . ^1H NMR spectra at 500 MHz were determined on a Varian VXR500 instrument. TLC analyses were on Whatman MK6F silica gel plates, with spots visualized under a 254-nm UV light. Products for elemental analyses were dried in vacuo at 70°C over P_2O_5 . Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA). 4-Amino-4-deoxy- N^{10} -methylpteroyl-L-lysine (**6**) (14a,b) and N^{10} -formylpteroyic acid (**15**) (18) were prepared as described earlier. N-Benzoyloxycarbonyl-L-aspartic acid was purchased from Bachem, Torrance, CA, and other chemicals from Aldrich, Milwaukee, WI. Microanalyses were carried out by Quantitative Technologies, Inc., Whitehouse, NJ, and were within 0.4% of theoretical values.

N^{10} -(4-Amino-4-deoxy- N^{10} -methylpteroyl)- N^E -(phosphonoacetyl)-L-lysine (**4**)

A suspension of phosphonoacetic acid (2.0 g, 0.014 mol) in freshly distilled SOCl_2 (65 ml) was stirred at 50°C (oil bath) for 3 h, then at 63°C for 4 h. Excess SOCl_2 was removed by rotary evaporation, and the last traces were co-distilled azeotropically with toluene to afford an oil, which was taken up in dioxane (28 ml), giving a solution assumed to contain 0.5 mmol/ml. This stock solution was used directly in the next step.

A suspension of **6** (50.7 mg, 0.1 mmol) in H_2O

(15 ml) was adjusted to pH 11.2 by dropwise addition of 1 N NaOH. When all the solid dissolved, the solution was cooled to 5°C and stirred with simultaneous dropwise addition of 1 N NaOH (3.75 ml) and a portion of the stock solution of phosphonoacetyl chloride in dioxane prepared above (2 ml, assumed to contain 1.0 mmol). Addition was performed in such a manner as to keep the pH between 7.0 and 9.5. The progress of the reaction was monitored by TLC (silica gel, 3:2:1 n-BuOH-H₂O-AcOH), which showed spots with R_f 0.15 (**4**), 0.30 (**6**), and 0.36. When reaction appeared to be complete, the solution was diluted to 10 ml and passed through a column of Sephadex G-10 (54×1.3 cm, swollen in H₂O from 24 g of dry powder) with H₂O as the eluent. Fractions were monitored by TLC (silica gel, 6:4:3 n-BuOH-H₂O-AcOH), and tubes showing a spot with R_f 0.25 were combined and freeze-dried to a yellow powder (235 mg) which was not fully desalted. The powder was suspended in H₂O (10 ml) at pH 6.5, a small amount of insoluble material was removed by filtration, and the filtrate was adjusted to pH 2.0 and filtered again. The filtrate was freeze-dried, and the residue was triturated successively with cold Me₂CO and MeOH to obtain a yellow powder (103 mg). This solid was dissolved in 3% NH₄HCO₃ (1.5 ml), the pH was adjusted to 11.0 with 28% NH₄OH, and the solution was applied onto a column of DEAE-cellulose (DE-52, HCO₃⁻ form, 1.3×17 cm) which was eluted successively with distilled H₂O (160 ml) and 3% NH₄HCO₃. Appropriate fractions of the latter eluent were combined and freeze-dried to obtain **4** as a pale-yellow solid (11 mg, 14%); mp >200°C dec; IR (KBr) ν 3400, 3150, 1640, 1610, 1550, 1515, 1405, 1265, 1210, 1055, 800 cm⁻¹.

C₂₃H₃₀N₉O₇P·0.4NH₃·10H₂O (M_r 767.6)

Calc C 36.22 H 6.78 N 17.27

Found C 36.24 H 6.39 N 17.41

4-Nitrophenyl Dimethoxyphosphonoacetate (**12**)

Trimethyl phosphonoacetate (9.10 g, 0.05 mol) was stirred and treated dropwise over 3 min with 1 N NaOH (50 ml, 0.05 mol). The solution was left at room temperature for 18 h and passed through a column of Dowex 50W-X2 (H⁺ form, 2×25 cm). The acidic fractions were combined and evaporated under reduced pressure. After several cycles of azeotropic distillation with toluene, dimethoxyphosphonoacetic acid was obtained as an oil (9.79 g); ¹H NMR (CDCl₃) δ 3.03 (d, J=

21 Hz, CH₂P), 3.85 (d, J=12 Hz, OMe), 6.55 (s, >2H, OH and H₂O). A solution of the crude acid in EtOAc (100 ml) was treated directly with p-nitrophenol (6.95 g, 0.05 mol) followed by DCC (10.3 g, 0.05 mol). The reaction mixture became warm, and TLC (silica gel, 1:1 acetone-ether) showed the formation of a new spot at R_f 0.5, along with unreacted p-nitrophenol (R_f 0.8). A second portion of DCC (5.15 g, 0.025 mol) was added, and when TLC showed that all the p-nitrophenol was consumed, the reaction was quenched by dropwise addition of 98% formic acid (1 ml). The solid was filtered and the filtrate was evaporated to obtain **12** as an oil which was used directly in the formation of **13** from **11** as described in the next step.

Methyl L-2-(N-Benzoyloxycarbonylamino)-3-N-(dimethoxyphosphonoacetyl)aminopropanoate (**13**)

A mixture of N^α-(benzyloxycarbonyl)-L-aspartic acid (**7**) (13.4 g, 0.05 mol), paraformaldehyde (2.5 g, 0.083 mol), and p-TsOH·H₂O (0.5 g) in toluene (100 ml) was heated to reflux in a Dean-Stark apparatus for 30 min and allowed to cool to room temperature. EtOAc was added to the two-phase mixture until it became homogeneous, and the solution was washed to neutrality with H₂O. The product was then extracted into 5% NaHCO₃, and the aqueous layer was acidified with HCl and extracted with EtOAc. The EtOAc layer was washed with H₂O and evaporated under reduced pressure to afford **8** as a thick oil from which the last traces of solvent were removed by heating in vacuo at 60°C; yield 13.6 g (98%). A dried sample of **8** (12.2 g, 0.044 mol) and DPPA (12.1 g, 0.044 mol) in t-BuOH (70 ml) containing Et₃N (6.12 ml, 4.44 g, 0.044 mol) was heated under reflux for 18 h. The solvent was evaporated under reduced pressure, the residue was partitioned between CHCl₃ and 5% NaHCO₃, and the CHCl₃ layer was washed with 1 N HCl and evaporated. The resulting crude **9** was taken up directly in MeOH (100 ml), and the solution was cooled to 0°C and stirred while adding NaOMe (2.7 g) in small portions over 5 min. After 15 min the reaction mixture was quenched with glacial AcOH (4 ml) and evaporated to dryness to obtain impure **10** (14.7 g) as an oil. A portion of this material (11.1 g) was purified by flash chromatography on silica gel (4×9 cm) with CHCl₃ as the eluent. Fractions containing a single TLC spot with R_f 0.6 (silica gel, CHCl₃) were pooled and evaporated to obtain pure **10** (6.64 g,

57%). Although others have reported this compound to melt at 88–90°C (23), we were unable to obtain it in a crystalline state. A solution of **10** (5.85 g, 0.0167 mol) in toluene (80 ml) containing *p*-TsOH·H₂O (3.04 g, 0.016 mol) was boiled in an open flask for 15 min to allow H₂O to escape, and the cooled reaction mixture was concentrated to dryness by rotary evaporation. The residue, consisting of the crude tosylate salt **11**, was dissolved directly in CH₂Cl₂ (40 ml) and to this solution was added a solution of **12** (4.91 g, 0.017 mol) in CH₂Cl₂ (40 ml) followed by Et₃N (7.8 ml, 7.0 g, 0.07 mol) in three portions while monitoring the reaction by TLC (silica gel, 1:1 acetone-Et₂O) for the disappearance of **12** (*R*_f 0.5) and formation of **13** (*R*_f 0.3). When the reaction was complete, the solution was washed with 1 N HCl and evaporated to dryness. The residue was purified flash chromatography on silica gel (4×10 cm) with 1:1 acetone-Et₂O as the eluent, and fractions giving a single TLC spot with *R*_f 0.3 were pooled and evaporated to obtain pure **13** as gum (2.56 g); IR (thin film) ν 3310, 3070, 3040, 3010, 2960, 2860, 1740–1710, 1655, 1545, 1440, 1420, 1265, 1040, 920, 880, 855, 815, 760, 705 cm⁻¹; ¹H NMR (CDCl₃) δ 2.85 (d, *J*=21 Hz, 2H CH₂P), 3.75 (m, 11H, CH₂N, 3 CH₃O), 4.45 (m, 1H, α -CH), 5.11 (m, 3H, benzylic CH₂ and NH), 6.25 (d, <1H, NH), 7.38 (s, 5H, Ph).

C₁₆H₂₃N₂O₈P (*M*_r 402.4)

Calc. C 47.77 H 5.76 N 6.96

Found C 48.02 H 5.74 N 6.90

3-(*N*-Phosphonoacetyl)amino-2-*L*-(*N*-pteroylamino)propanoic Acid (**5**)

A solution of **13** (804 mg, 2.0 mmol) in MeOH (25 ml) containing Me₃SiCl (381 μ l, 326 mg, 3.0 mmol), used as a convenient source of anhydrous HCl, was shaken with 5% Pd-C (60 mg) under 3 atm. of H₂ for 18 h. The catalyst was removed by filtration, and the solvent was evaporated to obtain crude **14**·HCl (610 mg, 100%) as a foam which was used directly in the next step; IR (KBr) ν 3430, 3270, 2970, 2860, 2670, 1755, 1675, 1550, 1445, 1395, 1325, 1245, 1190, 1035, 925, 875, 855, 815, 770, 715 cm⁻¹.

A suspension of N¹⁰-formylpteronic acid (**15**) (340 mg, 1.0 mmol) in dry DMF (10 ml) was treated with Et₃N (167 μ l, 121 mg, 1.2 mmol) and *i*-BuOCOCl (156 μ l, 164 mg, 1.2 mmol) and placed in an ultrasonication bath for 10 min until most of the solid dissolved. To the solution

was then added a solution of **14**·HCl (305 mg, 1.0 mmol) in dry DMF (4 ml) followed by Et₃N (139 μ l, 101 mg, 1.0 mmol). After two additional cycles of addition of *i*-BuOCOCl (65 μ l, 68 mg, 0.5 mmol) followed by Et₃N (69 μ l, 50 mg, 0.5 mmol) and **14**·HCl (151 mg, 0.5 mmol) followed by Et₃N (69 μ l, 50 mg, 0.5 mmol), the solution was added dropwise with stirring into H₂O (200 ml). The pH was adjusted to slightly below 7.0 with glacial AcOH, and the fine precipitate which formed was collected on a glass fiber filter. The solid was ground up, suspended in 2:1 CHCl₃-MeOH, and sonicated at 50°C until no more of it dissolved. After filtration to remove the insoluble material, the filtrate was diluted with Et₂O, and the precipitate was collected to obtain **16** as a light-yellow powder (117 mg); mp > 200°C dec; *R*_f 0.2, blue fluorescent spot (silica gel 2:1 CHCl₃-MeOH); IR (KBr) ν 3380, 2960, 2850, 1670, 1605, 1575, 1535, 1505, 1435, 1415, 1375, 1345, 1295, 1245, 1210, 1170, 1130, 1035, 975, 960, 945, 955, 880, 855, 825, 770 cm⁻¹; ¹H NMR (DMSO-*d*₆) δ 2.8–3.7 (complex m, 13H, side-chain CH₂N and CH₂P, 3 CH₃O), 4.35 (m, 1H, α -CH), 5.03 (m, 3H, 9-CH₂, NH), 6.77 (m, 1H, NH), 7.45–8.00 (m, phenyl), 8.5, (s, 1H, 7-H), 8.8 (m, N-CHO).

Compound **16** (56 mg, 0.095 mol) was suspended directly in dry DMF (2.5 ml) and kept at 50°C until all the solid dissolved. The solution was then cooled to room temperature, treated with Me₃SiBr (396 μ l, 459 mg, 0.03 mmol), and left to stand for 3 days. The excess Me₃SiBr was removed by rotary evaporation, the remaining DMF solution was treated with 1 N NaOH (10 ml), and after 15 min at room temperature the reaction was terminated by bringing the pH to 7.0 with HCl. Preparative HPLC batch purification on C₁₈ silica gel was performed with 4% MeCN in 0.05 M NH₄OAc, pH 6.9 as the eluent at a flow rate 10 ml/min. Two peaks were observed, with a peak height ratio of ca. 3:2 and elution times of 15 and 21 min, respectively. The slower peak was a minor but intensely UV-absorbing impurity, and was discarded. Pooled eluents containing the faster-moving peak were concentrated to dryness by rotary evaporation and two cycles of lyophilization to obtain **5** as a pale greenish-yellow solid (23 mg); mp >200°C dec; IR (KBr) ν 3410, 1610, 1545, 1515, 1405, 1340, 1310, 1265, 1195, 1180, 1135, 1065, 915, 840, 825, 770 cm⁻¹; ¹H NMR (DMSO-*d*₆, 500 MHz) δ 3.40 (H₂O obscuring the side-chain CH₂N and CH₂P), 4.15 (m, 1H, α -CH), 4.44 (s, 2H, 9-CH₂), 6.60

(d, 2H, 3'- and 5'-H), 6.86 (m, 1H, NH), 6.95 (broad s, 2H, NH₂), 7.58 (d, 2H, 2'- and 6'-H), 7.80 (m, 1H, NH), 8.07 (m, 1H, NH), 8.62 (s, 1H, 7-H).

C₁₉H₂₁N₈O₈P·NH₃·4.5H₂O (*M_r* 618.6)

Calc C 36.90 H 5.38 N 20.38

Found C 36.54 H 5.05 N 20.69

3-*N*-(Methoxyphosphono)acetyl-amino-2-*L*-(*N*-pteroylamino)propanoic Acid (17**)**

A solution of crude **16** (113 mg, 0.192 mmol) in DMSO (2 ml) was stirred and treated dropwise over 3 min with 1 N NaOH (2 ml, 2 mmol). The clear solution was kept at 60°C in a water bath for 5 min, then cooled, diluted with H₂O (15 ml), and adjusted to pH 7 with 10% AcOH. Preparative HPLC (C₁₈ silica gel, 5% MeCN in 0.05 M NH₄OAc, pH 6.9, 10 ml/min) showed two peaks (5:1 ratio) with elution times of 7 min and 9 min, respectively. Pooled fractions containing only the larger, faster-moving peak were collected, concentrated by rotary evaporation, and freeze-dried. The residue was taken up in H₂O with addition of a few drops of 28% NH₄OH. Freeze-drying afforded **17** as a pale-yellow solid (69 mg, 58%); mp >200°C dec; IR (KBr) ν 3430br, 2860, 1685, 1645, 1610, 1575, 1545, 1520, 1450, 1405, 1340, 1310, 1265, 1200, 1135, 1070, 1050, 975, 840, 825, 770, 740 cm⁻¹; ¹H NMR (D₂O, 500 MHz) δ 2.77 (d, *J*=20 Hz, 2H, CH₂P), 3.52 (m overlapping a doublet with *J*=10 Hz, 5H, POMe and β -CH₂), 4.37 (m, overlapping a broad H₂O peak), α -CH and CH₂N, 6.53 (d, *J*=8 Hz, 2H, 3'- and 5'-H), 7.55 (d, *J*=8 Hz, 2'- and 6'-H), 8.62 (broad s, 1H, 7-H).

C₂₀H₂₃N₈O₈P·NH₃·3.5H₂O (*M_r* 614.6)

Calc C 39.09 H 5.41 N 20.51

Found C 38.92 H 5.40 N 20.45

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