Prodrug Forms of *N*-[(4-Deoxy-4-amino-10-methyl)pteroyl]glutamate-γ-[ψP(O)(OH)]-glutarate, a Potent Inhibitor of Folylpoly-γ-glutamate Synthetase: Synthesis and Hydrolytic Stability

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Ester prodrugs of the phosphinate pseudopeptide *N*-[(4-deoxy-4-amino-10-methyl)pteroyl]glutamate-γ-[ψP-(O)(OH)]-glutarate (**1a**) were synthesized. H-phosphinic acids derived from N-Cbz vinyl glycine esters were converted to the desired pseudopeptides by Michael addition to α-methyleneglutarate esters. Pivaloyloxymethyl (POM) ester moieties were incorporated in both the N-terminal and C-terminal fragments prior to formation of either C−P bond. *N*-Alkylation of the corresponding amides derived from *p*-(*N*-methyl)-aminobenzoic acid with 2,4-diamino-6-(bromomethyl)pteridine gave the target compounds. POM esters of methotrexate and the corresponding γ-glutamyl conjugate were also synthesized using the same strategy. All prodrugs were evaluated in Chinese hamster ovary cells. Although the pseudopeptide prodrugs were ineffective, prodrugs of methotrexate and the corresponding γ-glutamyl conjugate were equipotent with the parent compounds. Stability of the prodrugs was investigated in both phosphate buffer and cell line medium to provide a rationale for the observed biological data.

Introduction

Folic acid (pteroyl-L-glutamate, PteGlu) is an essential vitamin which, when reduced to (6*S*)-5,6,7,8-tetrahydrofolic acid (H_4-PteGlu), leads to one-carbon donors involved in the biosynthesis of serine, glycine, methionine, and thymidylate and also in de novo synthesis of purines.[1] Due to the critical functions of folic acid in human metabolism, numerous antifolates have been designed and synthesized as drugs for chemotherapy of malignant diseases, such as leukemia[2] and solid tumors,[3−5] as well as for nonmalignant diseases such as rheumatoid arthritis and psoriasis.[6] In most cells, both folates and antifolates occur exclusively as their polyglutamate conjugates,[7] which are formed in a reaction catalyzed by folylpoly-γ-glutamate synthetase (FPGS, EC 6.3.2.17).[8] FPGS catalyzes an ATP-dependent ligation of glutamic acid to reduced folates, including H_4PteGlu, as well as anticancer drugs, such as methotrexate (MTX, AMPteGlu, **2a**), 5,10-dideazatetrahydrofolate, and related derivatives. There are at least two contributions of FPGS-catalyzed biosynthesis of polyglutamate derivatives to cell viability. First, because these conjugates cannot exit the cell,[9] the intracellular concentration of folates and antifolates (∼10 μM) are much higher than that found in the extracellular compartments (∼10 nM).[7] Second, the polyglutamate derivatives of folates or antifolates are usually better substrates or inhibitors than the parental monoglutamates in a variety of folate-dependent reactions.[8] A Chinese hamster ovary (CHO) cell line that transports monoglutamate folates normally, but cannot synthesize folylpolyglutamates, is unable to survive even in the presence of supraphysiological levels of reduced monoglutamate folates.[10] Accordingly, specific inhibition of FPGS, resulting in decreased levels of the polyglutamate conjugates, should lead to a block of folate-dependent, one-carbon biosynthetic reactions and a cessation of DNA replication in the tumor cells.[4,11] In addition, resistance to several antifolates has been attributed to reduced FPGS activity in several tumor-derived cell lines.[12] Thus, FPGS is a new target for cancer chemotherapy to introduce selective folate deficiency and/or attack tumors that have developed resistance to antifolates due to the reduced activity of FPGS.[4,11]

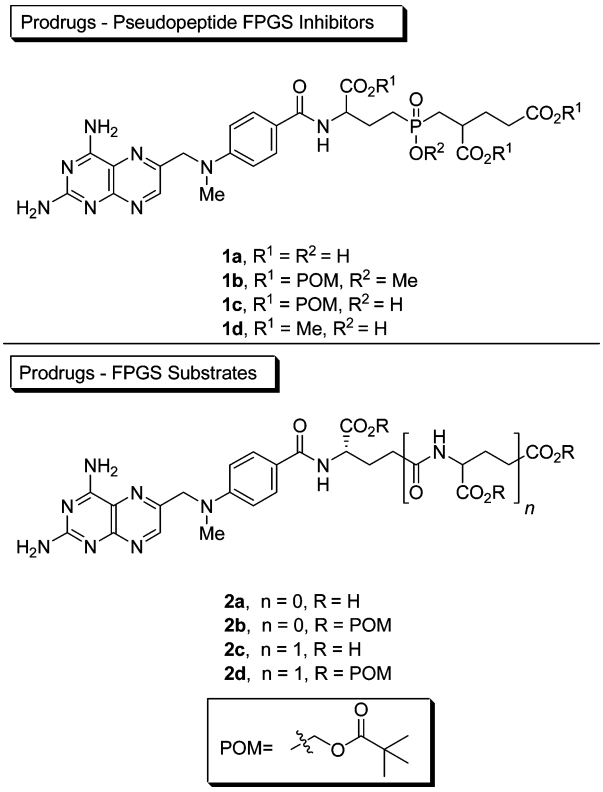

Figure 1. Free acid and prodrug forms of AMPte-Glu-γ-{ψP(O)(OH)}-Glu (**1a**−**d**), AMPte-Glu (**2a**, **2b**), and AMPte-Glu-γ-Glu (**2c**, **2d**).

On the basis of the mechanism of FPGS-catalyzed ligation of glutamic acid to reduced folates and antifolates,[13] we previously designed and synthesized a phosphorus-containing pseudopeptide analogue of AMPteGlu-γ-Glu (**2c**), *N*-[(4-amino-4-dexoxy-10-methyl)pteroyl]-L-glutamyl-γ-[Ψ{P(O)(OH)CH_2}]-glutarate (AMPteGlu-γ-[Ψ{P(O)(OH)CH_2}]-glutarate, **1a**, Figure 1),[14−16] to mimic the tetrahedral intermediate formed during the ATP-dependent reaction. This compound is the most potent in vitro inhibitor of FPGS described to date based upon the

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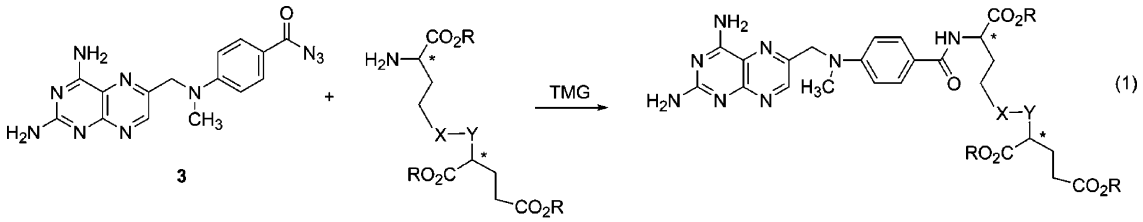

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4a, X-Y = P(O)(OH)CH$_2$, R = H
4b, X-Y = P(O)(OMe)CH$_2$, R = POM
4c, X-Y = P(O)(OH)CH$_2$, R = POM
4d, X-Y = P(O)(OH)CH$_2$, R = Me
4e, X-Y = C(O)NH, R = H
4f, X-Y = C(O)NH, R = POM

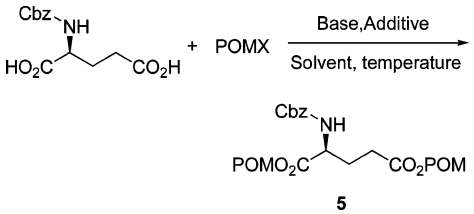
1a, X-Y = P(O)(OH)CH$_2$, R = H
1b, X-Y = P(O)(OMe)CH$_2$, R = POM
1c, X-Y = P(O)(OH), R = POM
1d, X-Y = P(O)(OH), R = Me
2c, X-Y = C(O)NH, R = H
2d, X-Y = C(O)NH, R = POM

heterocyclic pharmacophore 2,4-diaminopteridine. Unfortunately, when used as an inhibitor of cell growth in human cancer cells (CCRF-CEM), the phosphinic MTX analogue **1a** was completely ineffective,[15] presumably due to its highly hydrophilic structure and inability to act as a substrate for either of the folate transporters reduced folate carrier (RFC) or the folate binding protein (FBP) to penetrate the cell membrane.[17] To overcome this transport barrier, a prodrug strategy was adapted and investigated as an alternative approach for intracellular FPGS inhibition, which could ultimately be lethal to tumor-derived cell lines.

Prodrugs are derivatives of drug molecules that require a chemical or enzymatic transformation in order to release the pharmacologically active drug within cells. To date, prodrug strategies have proved to be an important method in pharmacology for the design and modification of drug candidates, because they can result in better transport, uptake, and metabolism, all of which are very important factors, together with the bioactivity of the parent compound, in determining ultimate drug efficacy. In preclinical and clinical studies, increasing the membrane permeability of polar and hydrophilic drug candidates with good inhibitory potency has been widely investigated.[18−24] For those candidates with acid functionalities, which have low cell permeability and could therefore cause drug delivery problems, masking the hydrophilic moieties results in a significant increase of the lipophilicity, which can make them good substrates for membrane transport.[25] Numerous reports in the literature, including clinical studies leading to commercial drugs, have shown the value of using esters as prodrugs, among which pivaloyloxymethyl (POM) esters are particularly attractive, because they are very hydrophobic and are hydrolyzed (enzymatic or nonenzymatic) to the parent acid in an irreversible reaction.[26−31] Since the membrane transport barrier is presumably a major reason the antifolate analogue **1a** is ineffective as an inhibitor of cell growth,[15] converting the phosphinic pseudopeptide **1a** to prodrug esters may be an effective solution to overcome the transport barrier problem.[32] Herein, we report the syntheses of POM ester (**1b** and **1c**) and methyl ester (**1d**) prodrugs of the phosphinic pseudopeptide **1a** (Figure 1) and an investigation of their chemical stabilities in phosphate buffer and cell line medium. Because FPGS inhibitor **1a** is based on the MTX heterocycle and the pharmacology of MTX has been investigated extensively,[2,6,33] two reference compounds, **2b** and **2d**, prodrug esters of MTX (AMPte-Glu, **2a**) and its γ-glutamyl conjugate (AMPte-Glu-γ-Glu, **2c**), were also synthesized.

The main transport protein involved in the cellular uptake of MTX and related compounds is the reduced folate carrier (RFC). On the basis of the ideas discussed above, the prodrug esters should not require functional RFC for cell uptake and would cross the cell membrane by passive diffusion. However, MTX (**2a**) requires RFC for uptake, and although not as extensively studied, AMPteGlu-γ-Glu (**2c**) is not a good RFC ligand and is not effectively taken up by cells[34,35] in the absence of hydrolysis by secreted γ-glutamyl hydrolase.[36,37] The POM esters and their parent acids, **1** and **2**, were evaluated in wild-type CHO cells as well as a MTX-resistant mutant[38] and a "rescued" CHO cell line arising from transfecting the MTX-resistant mutant with DNA coding for human RFC.[39]

Table 1. Conditions for the Preparation of *N*-Cbz-glutamic Acid Di-POM Ester[a]



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entry	X (equiv)	base (equiv)	additive (equiv)	solvent	temp	yield (%)[b]
1	Cl (2.0)	NaOH (2.2)	$AgNO_3$ (1.0)	DMF	rt	13
2	Cl (2.2)	K_2CO_3 (2.5)	$AgNO_3$ (2.2)	DMF	rt	10
3	Cl (2.2)	K_2CO_3 (2.5)	NaI (2.5)	DMF	rt	27
4	Cl (3.0)	DBU (3.0)	DMAP (0.2)	THF	reflux	28
5	Cl (4.0)	K_2CO_3 (2.5)	NaI (4.0)	acetone	reflux	45
6	Cl (4.0)	DBU (2.5)	DMAP (0.2) NaI (4.0)	dioxane	reflux	45
7	I (2.2)	K_2CO_3 (2.5)	-	DMF	rt	24
8	I (5.0)	DBU (3.0)	DMAP (0.3)	dioxane	reflux	24
9	I (5.0)	Ag_2CO_3(5.0)	-	dioxane	reflux	15
10	I (5.0)	Ag_2CO_3(3.0)	-	DMF	rt	71
11	I (5.0)	Ag_2CO_3(3.0)	$(MeO)_2P(O)Me$ (5)	DMF	rt	80
12	I (20)	Ag_2CO_3 (5.0)	$(MeO)_2P(O)Me$ (20)	DMF	rt	92

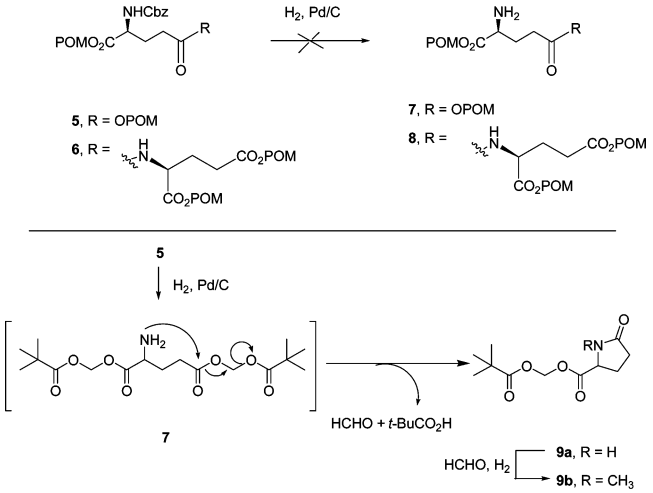
[a] All of the reactions proceeded overnight. [b] Isolated yield.

Results and Discussion

In our previous work, **1a** was synthesized successfully by coupling of the free phosphinic acid **4a** and acyl azide **3** (eq 1) which, in turn, was obtained from a pteroic acid analogue derived from the coupling of 2,4-diamino-6-(bromomethyl)-pteridine and *N*-methyl-*p*-aminobenzoic acid.[14,16,40] The synthesis of 6(*S*)- and 6(*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolic acid and its poly-γ-glutamate derivatives have recently been reported using similar methods.[41] Accordingly, based on the same strategy, target compounds **1b-d** and **2d** should be accessible as well by reaction of azide **3** with POM esters **4b**−**d** and **4f** in the presence of an appropriate base. POM ester **2b**, a derivative of MTX should also be available via coupling of **3** with glutamate di-POM ester (**7**).

As a key step, preparation of POM ester of *N*-Cbz-glutamic acid using a POM halide was then investigated (Table 1).[32,42] When using POM chloride and *N*-Cbz glutamic acid as the substrates, the yields were consistently low under several conditions (Table 1, entries 1−4), even in the presence of

Scheme 1

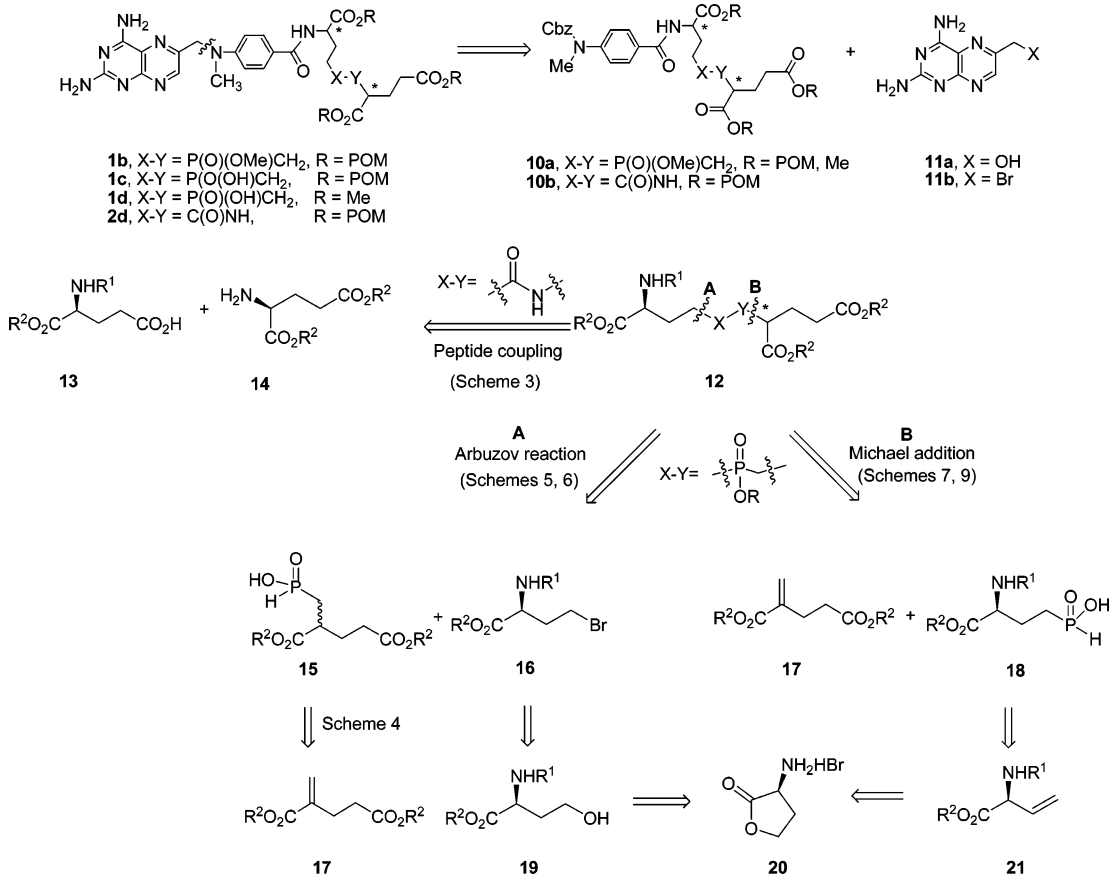


catalysts such as silver nitrate and DMAP. Only when sodium iodide was included to effect in situ generation of POM iodide, together with an increase in the reaction temperature, were modest yields (45%) of the desired product obtained (Table 1, entries 5, 6). When POM iodide (POMI)[43] was employed directly, an excellent yield was achieved when the iodide and the additive dimethyl methylphosphonate[32] were both present in large excess (Table 1, entry 12). When less iodide and phosphonate were used, the yield was lower but still good (Table 1, entry 11). If the additive was not utilized, the yield of the reaction decreased even further (Table 1, entry 10). The use of POMI under other conditions led to unsatisfactory yields (Table 1, entries 7−9).

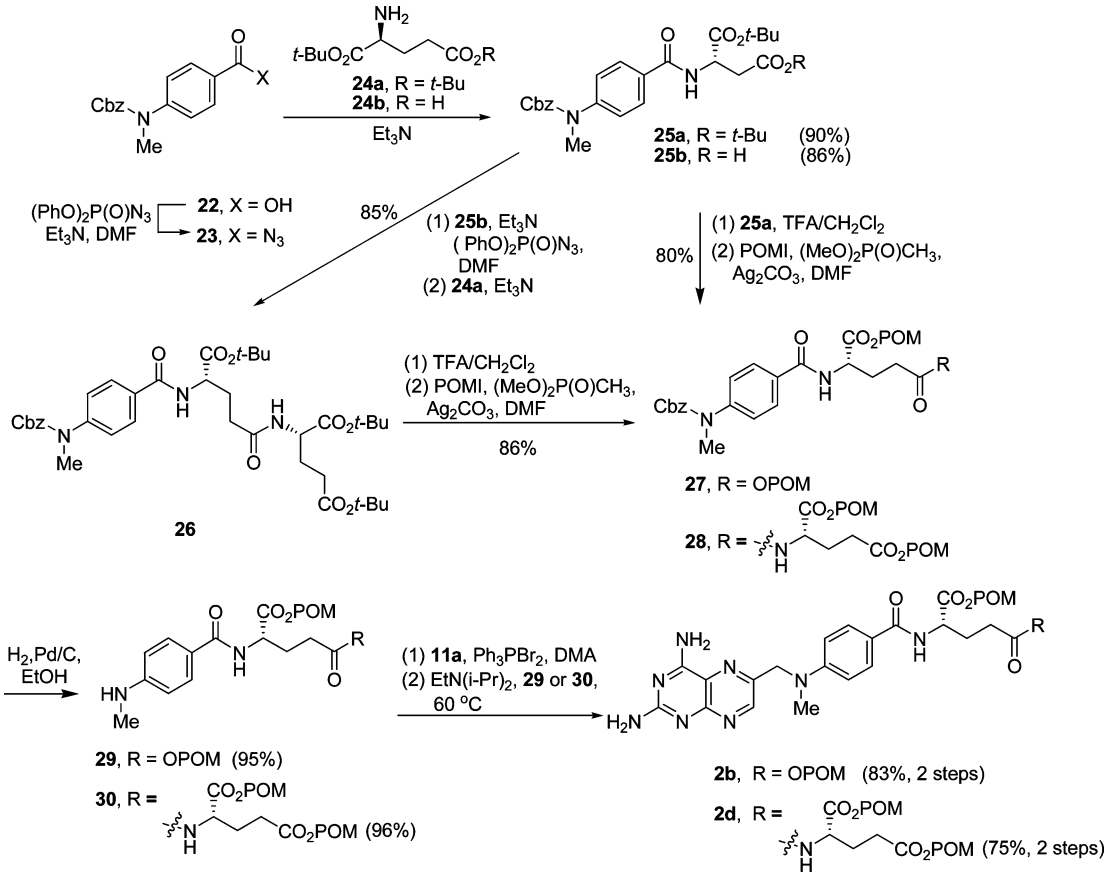
With di-POM *N*-Cbz-glutamate **5** in hand, the synthesis of L-glutamic acid bis-POM ester, **7**, was investigated (Scheme 1). Unfortunately, efforts to remove the Cbz group from the amino group of the POM ester **5** were unsuccessful. No desired product was obtained. Instead, pyroglutamate derivatives **9a** and **9b** were isolated and identified. That suggested that although the desired free amine di-POM ester **7** was formed in the hydrogenolysis, it was not stable. Immediately following its formation, intramolecular reaction of the amine and POM ester gave the more stable cyclized product, lactam **9a**, part of which reacted further with the formaldehyde generated in situ and was then reduced to yield the *N*-methylated derivative **9b**. The use of Boc_2O to trap the free amine as formed during hydrogenolysis was investigated. Unfortunately, the pyroglutamate derivatives **9a** and **9b** were still the major products, with only 19% of the desired *N*-Boc-protected di-POM glutamate obtained. Regardless of the low yield and the byproducts, the small amount of *N*-Boc-protected POM ester was converted to the amine TFA salt (**7**·TFA) and allowed to react with the azide **3** in the presence of triethylamine. However, this attempted synthesis of POM ester **2b** was unsuccessful and none of the desired product was observed. The POM ester of the γ-glutamyl dipeptide **6** was also prepared. Unfortunately, similar to the *N*-Cbz-glutamic POM ester **5**, removal of the Cbz from the amino group was also unsuccessful. After hydrogenolysis, multiple spots were observed on TLC analysis, indicating probable intermolecular reaction of the amine with the POM ester moiety. This approach was abandoned due to the lability of free amines **7** and **8**.

Another approach to the prodrugs was then pursued, and the retrosynthetic route is illustrated in Scheme 2. In this approach, 2,4-diamino-6-(bromomethyl)pteridine **11b** is used instead of pteroyl azide **3** to introduce the heterocycle. This strategy avoids

Scheme 2



Scheme 3



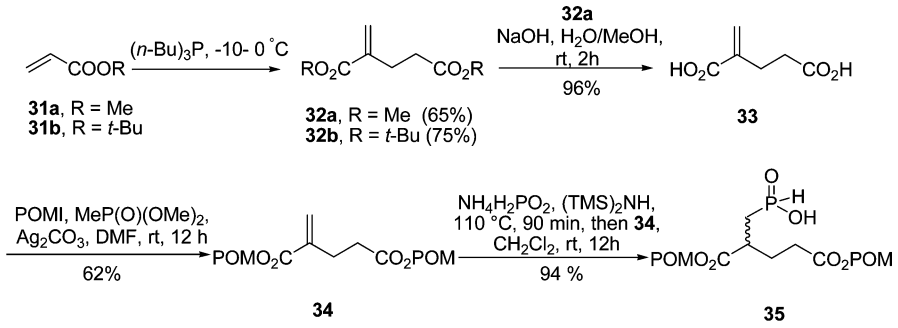
the generation of free amino acid POM esters that would react as described above (Scheme 1). Although the secondary amine of *N*-Me-*p*-ABA might also be problematic in terms of possible reaction with the POM esters, the low nucleophilicity of the aromatic amine as well as steric hindrance of the adjacent phenyl and methyl groups should prevent inter- or intramolecular reaction of the amine and POM. The *N*-methyl *p*-aminobenzoyl-protected POM ester of the peptide or pseudopeptide **10** could be prepared in several ways. In the case of AMPte-Glu-(α-POM)-γ-Glu-(α,γ-di-POM) (**2d**), synthesis of the *N*-acylated γ-glutamyl peptide would be effected through standard solution-phase peptide coupling reactions,[44] followed by conversion to the corresponding tri-POM ester. In the case of **1b,c**, the prodrug esters of AMPteGlu-γ-[Ψ{P(O)(OH)CH_2}]-glutarate (**1a**), two routes are available. One is through an Arbuzov reaction of the 2-amino-4-bromobutyrate **16** with phosphinic acid **15** (route A).[45,46] Phosphinic acid **15** could be obtained via a Michael addition of $(TMSO)_2PH$ to α-methyleneglutarate **17**.[47,48] Bromide **16** could be prepared from 2-amino-4-hydroxybutyrate **19**, which is derived from 2-aminobutyrolactone **20**. The other route is through a Michael addition of the phosphinic acid **18** to α-methyleneglutarate **17** (route B). Phosphinic acid **18** could be synthesized from vinylglycine **21** by a radical reaction with ammonium hypophosphite under Montchamp's condition.[16,49] *N*-Protected vinylglycine **21** could be derived from aminobutyrolactone **20** following a procedure developed by our group.[16,50]

Accordingly, the syntheses of prodrug esters **1** and **2** were then pursued. POM ester **2b** was first synthesized starting from acid **22**, as shown in Scheme 3. By using the same conditions used previously to synthesize pteroyl azide **3**, acid **22** was converted in situ to the corresponding azide **23**,[14,51] which was then allowed to react with di-*tert*-butyl L-glutamate (**24a**) to give compound **25a**. By treatment with TFA, the two *tert*-butyl groups on **25a** were removed to afford the corresponding free acid, which was converted to the POM ester **27** was then deprotected by hydrogenolysis to give **29**. 2,4-Diamino-6-(bromomethyl)pteridine **11b** was synthesized in situ by reaction of 2,4-diamino-6-(hydroxymethyl)pteridine hydrochloride **11a** with dibromotriphenylphosphine.[14] Upon addition of **29** to **11b** in the presence of Hünig's base, POM ester **2b** was obtained in good yield.

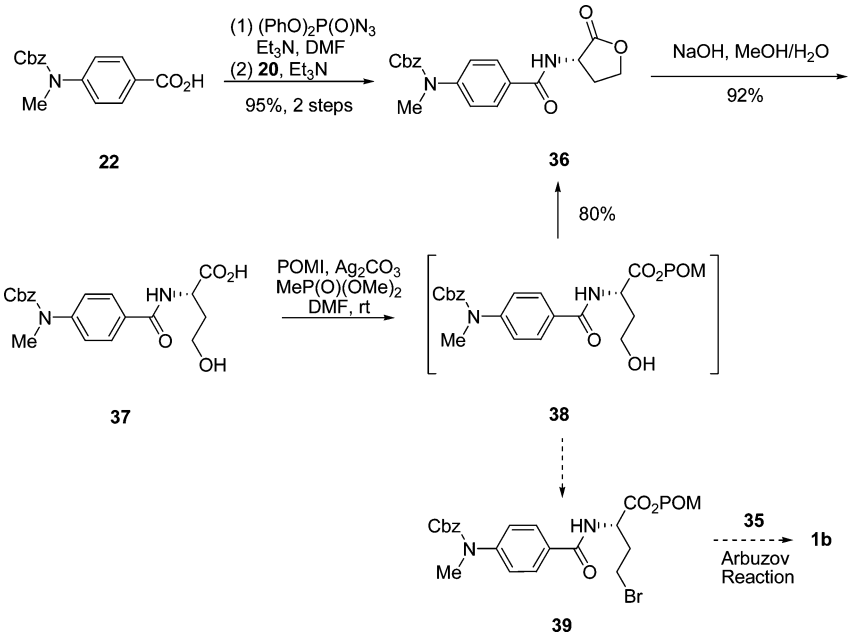
Encouraged by the successful synthesis of **2b**, synthesis of our second target compound, **2d**, the tris-POM ester of AMPte-Glu-γ-Glu (**2c**), was then investigated. Acid **22** was converted to the corresponding azide **23** and then reacted with the α-*tert*-butyl glutamate **24b** in the presence of tetramethylguanidine (TMG) to give acid **25b**, which was then converted to the corresponding acyl azide by reaction with diphenylphosphoryl azide. Reaction of the resulting azide and di-*tert*-butyl glutamate (**24a**) afforded tri-*tert*-butyl ester **26** in excellent yield. Treatment of **26** with TFA gave the triacid, which was esterified to the corresponding POM ester **28** under the standard conditions. The Cbz group of tris-POM ester **28** was removed by hydrogenolysis to give the coupling partner **30**. The resulting secondary amine was allowed to react with **11b**, generated in situ from **11a**, to give the AMPteGlu-γ-Glu-tri-POM ester **2d** in good yield.

With the successful synthesis of prodrug esters **2b** and **2d** completed, a similar approach to synthesize the prodrug esters of AMPteGlu-γ-[Ψ{P(O)(OH)CH_2}]-glutarate was then investigated, initially via using the Arbuzov reaction (Scheme 2, route A). Accordingly, efforts were made to synthesize the phosphinic acid **35** (Scheme 4). The dimer of methyl acrylate **32a** was first prepared following a literature procedure in which hydroquinone was used as an additive.[52] However, the yield of the desired dimer was quite low (ca. 30%). Since hydroquinone usually is used to prevent polymerization reactions, we carried out the

Scheme 4



Scheme 5



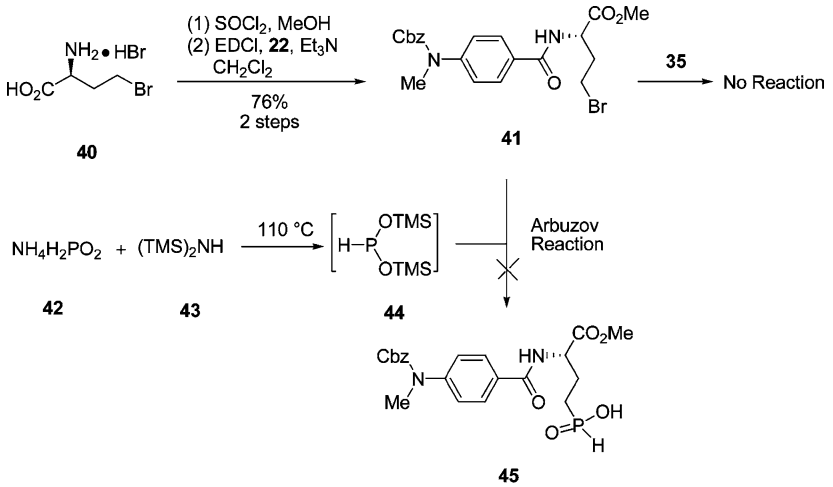
dimerization in its absence with encouraging results. Neat methyl acrylate was treated with tri-*n*-butyl phosphine at low to ambient temperature to yield the dimerized methyl ester **32a** in an acceptable yield, the best yield reported to date. Under the same conditions, di-*tert*-butyl α-methyleneglutarate **32b** was also synthesized in good yield. Dimer **32a** was then hydrolyzed to give the free acid **33** in nearly quantitative yield.[52] Upon treatment of acid **33** with the POMI under the standard esterification conditions, di-POM α-methyleneglutarate **34** was obtained in modest yield. Subsequently, reaction of the POM ester **34** with the P^{III} species generated in situ under Regan's conditions[48] gave the desired phosphinic acid **35** in excellent yield.

With the phosphinic acid **35** in hand, synthesis of bromide **39**, the other component for the Arbuzov reaction, was investigated (Scheme 5). Azide **23** was generated in situ from acid **22** as described above (Scheme 3) followed by reaction with α-aminobutyrolactone **20** to give lactone **36** in excellent yield. The lactone was then hydrolyzed by treatment with sodium hydroxide to afford the γ-hydroxybutyric acid **37**, a proposed precursor of POM ester **39**. Unfortunately, after **37** was treated with POMI under the standard conditions, no POM ester **38** was isolated. Instead, reversion to lactone **36** was observed. A possible explanation may be that acid **37** was indeed esterified to the corresponding POM ester **38**. However, under basic conditions, the hydroxy group at the γ-position attacked the POM ester moiety to yield the lactone **36**. This facile intramolecular reaction is reminiscent of what was observed during attempted removal of the Cbz group from *N*-Cbz-glutamic acid bis-POM ester **5** (Scheme 1).

In addition to pursuing the synthesis of γ-bromo-POM ester **39**, we also investigated conditions to realize the Arbuzov reaction between a P^{III} species and a related γ-bromo ester **41** (Scheme 6). Ester **41** was first synthesized by coupling acid **22** with the methyl ester of 2-amino-4-bromobutyric acid. However, reaction of the phosphinic acid **35** with the methyl ester **41** under Regan's conditions[48] failed to provide the desired pseudopeptide. A model reaction was then investigated using a smaller P^{III} species, **44** (prepared in situ by the reaction of ammonium hypophosphite and hexamethyldisilazine), which was combined with alkyl bromide **41**. Unfortunately, under several different conditions, the desired Arbuzov substitution reaction was not observed (Scheme 6). These failures are not surprising on the basis of several examples in the literature that document intramolecular attack of the amide carbonyl group at a distal carbon bearing a good leaving group.[46,53,54] In the present case, intramolecular cyclization presumably occurred in which the amide carbonyl of **39** or **41** attacked the γ-bromide. On the basis of these results, this approach was abandoned.

Therefore, an alternate approach (Scheme 2, route B) to the target phosphinate esters via Michael addition of a phosphinic acid to an α-methyleneglutarate was explored starting from *N*-Cbz-vinylglycine esters **46** (Scheme 7).[16] To establish the viability of this approach in the synthesis of ester derivatives of pseudopeptide **1a**, synthesis of methyl ester **1d** was explored. Formation of the first phosphorus−carbon bond was effected

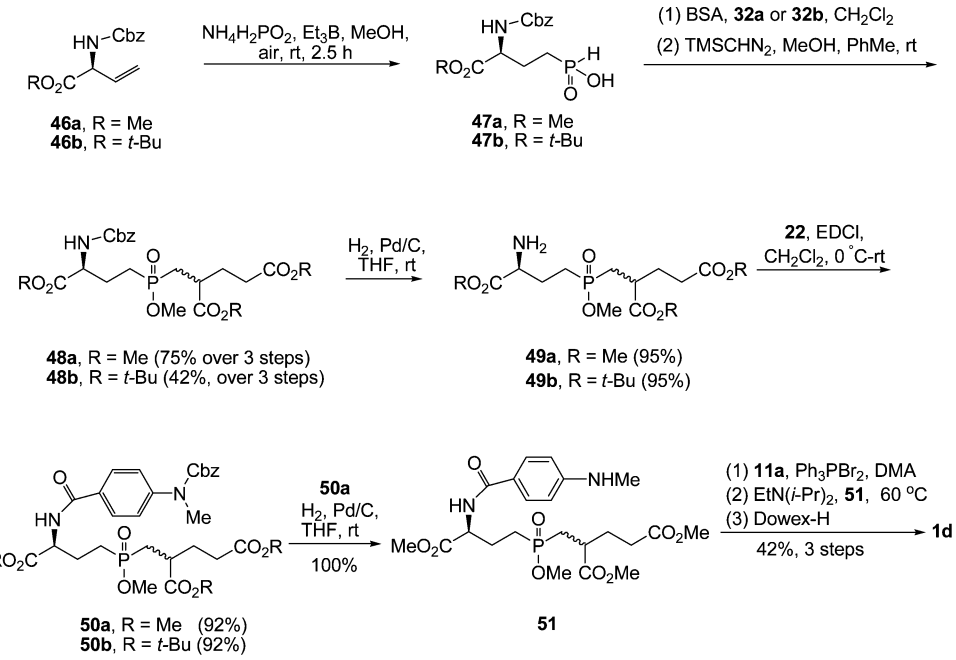
Scheme 6



Entry	Conditions
1[a]	CH_2Cl_2, rt, overnight
2[a]	*n*-Bu_4NI (0.1 eq), CH_2Cl_2, reflux, overnight
3	Toluene, reflux, overnight

[a] Before the bromide was added, ammonium hypophosphite and $(TMS)_2NH$ were heated at 105~110°C for 90 minutes.

Scheme 7



by reaction of the vinylglycine methyl ester **46a** with ammonium hypophosphite in the presence of triethylborane open to the air for several hours.[16] Addition of the resulting phosphinic acid **47a** to the Michael acceptor, dimethyl α-methyleneglutarate **32a**, using BSA as the P^{III} initiator afforded the *N*-Cbz-protected phosphorus-containing pseudopeptide, which was methylated by trimethylsilyl diazomethane ($TMSCHN_2$)[55] to give the tetramethyl ester **48a**.[16] Removal of the Cbz group on the amino group of **48a** by hydrogenolysis yielded the free amine **49a**, which was allowed to couple with acid **22** to afford the key intermediate **50a** for the prodrug ester **1d**, and a possible precursor to prodrug esters **1b** and **1c**.

Removal of the Cbz group of the tetramethyl ester of the phosphinate pseudopeptide **50a** by hydrogenolysis in THF gave the secondary amine precursor **51**, which was allowed to react with pteridinylmethyl bromide **11b** generated in situ from **11a**. Interestingly, the isolated product was not the expected tetramethyl ester but the diisopropylethylamonium salt of the phosphinic acid (P−OH), in which the methyl ester (P−OMe) was removed. The salt was converted to the desired phosphinic acid **1d** by treatment with Dowex-H.

Demethylation of the phosphinic methyl ester may be due to the presence of bromide ion formed in the reaction, since phosphonic acid alkyl esters can be dealkylated by halide anion (Figure 2, path A).[56,57] However, the use of AgOTf as a bromide ion scavenger failed to intercept the demethylation reaction. Because the amount of the dibromotriphenylphosphine used in the in situ formation of **11b** from **11a** was in excess, it is

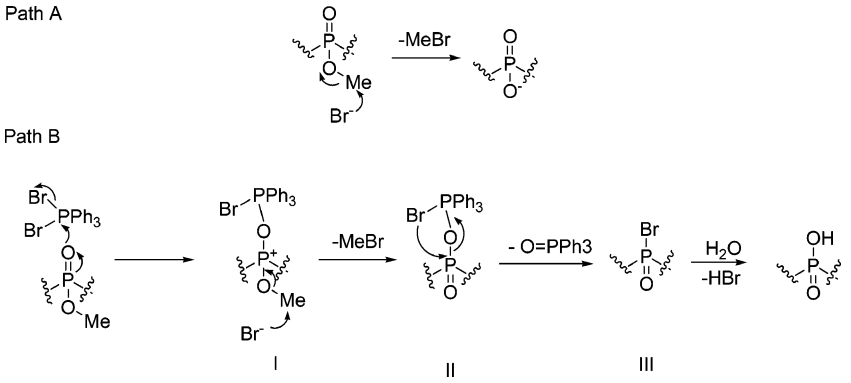
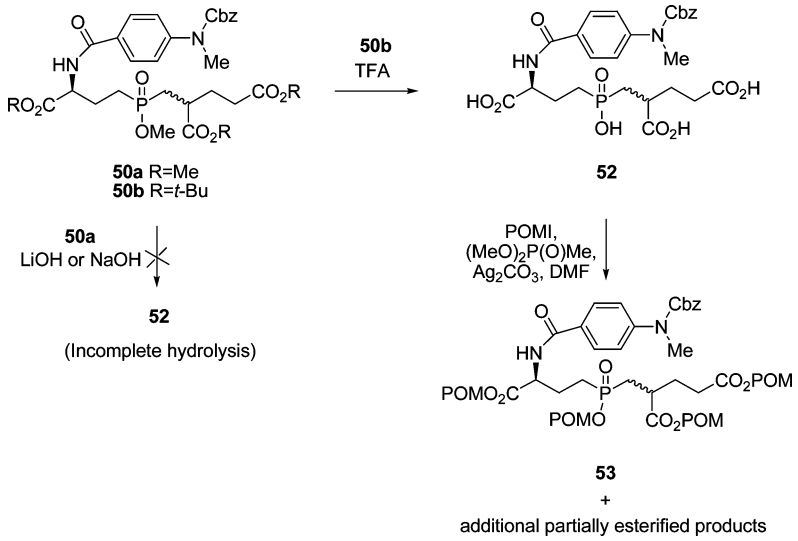


Figure 2. Possible mechanisms of demethylation of phosphinic acid P-O-methyl esters.

Scheme 8

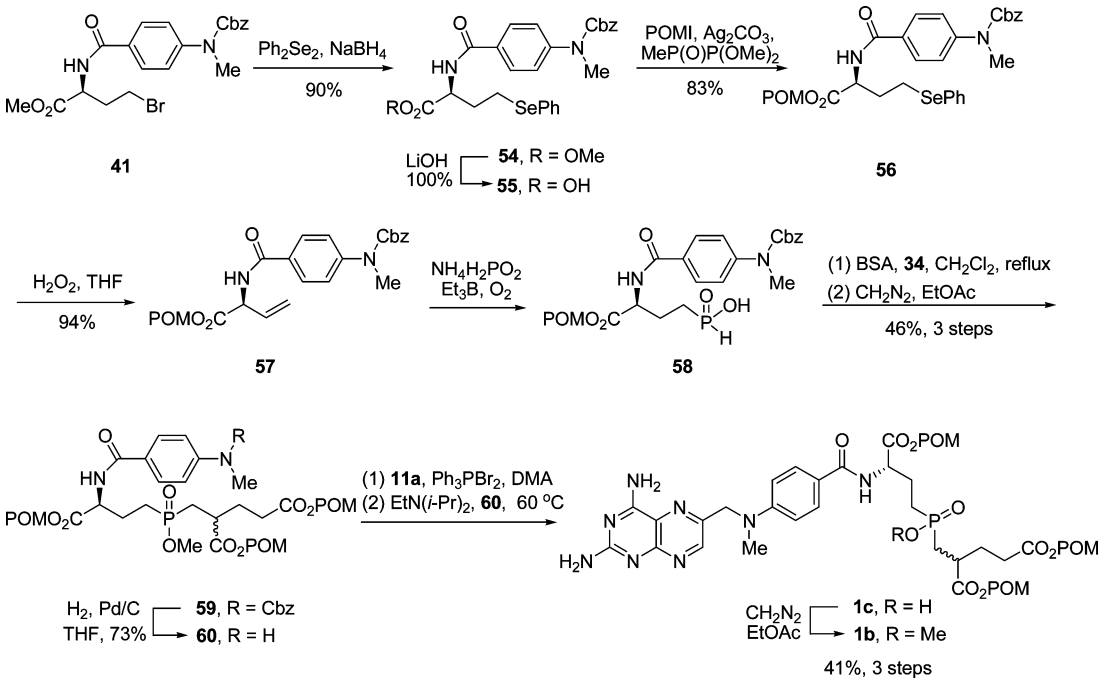


possible that the reaction proceeded via an alternate mechanism. (Figure 2, path B). Which of the two pathways is operative remains to be determined.

Encouraged by the successful synthesis of the methyl ester **1d**, research on the synthesis of POM ester **1b** was initiated. With the tetramethyl phosphinic pseudopeptide **50a** in hand, hydrolysis of the methyl esters to give the corresponding tetraacid, for conversion to the desired POM ester **53** was investigated (Scheme 8). However, hydrolysis of the tetramethyl ester to its free acid proved to be surprisingly slow. Several attempts using lithium hydroxide or sodium hydroxide in a mixture of methanol and water failed, either at room temperature or at reflux. ^{1}H NMR spectra of the isolated products indicated incomplete hydrolysis of the methyl esters. Initially, it was thought that the phosphinic acid methyl ester was the most difficult to hydrolyze. However, after the methyl ester of the phosphinic group was hydrolyzed using thiophenol and triethylamine,[58,59] further hydrolysis of the resulting trimethyl ester still could not be completed. Because the completely hydrolyzed and incompletely hydrolyzed products were difficult to separate and the nonhydrolyzed sites were difficult to determine, use of the crude hydrolysis product to prepare the corresponding POM ester is untenable.

Considering the fact that the carboxylic methyl esters cannot be hydrolyzed completely under standard basic conditions, but that the phosphinic acid methyl ester can be removed during the subsequent coupling reaction with 6-(bromomethyl)pteridine **11b** (Figure 2), another approach to the synthesis of POM ester **53** was pursued. The use of a *tert*-butyl ester precursor instead of a methyl ester was chosen since the *tert*-butyl groups can be easily removed with an acidic reagent, such as TFA. Following the procedure employed to synthesize tetramethyl pseudopeptide **50a**, the corresponding tris-*tert*-butyl ester **50b** was synthesized (Scheme 7) starting with *N*-Cbz-vinylglycine *tert*-butyl ester **46b** (see Supporting Information).[60,61] Treatment of **46b** with ammonium hypophosphite and triethylborane in methanol open to air afforded the phosphinic acid **47b**. Compound **47b** was then allowed to react with BSA to form the corresponding nucleophilic P^{III} species followed by reaction with di-*tert*-butyl α-methylene glutarate **32b** to afford the corresponding tri-*tert*-butyl phosphinic acid, which was reacted with $TMSCHN_2$ to give the desired pseudopeptide **48b**. The Cbz group was then exchanged for *N*-Me-*p*-ABA simply by hydrogenation and coupling with acid **22** in the presence of EDC to provide the desired key intermediate **50b**. Surprisingly, treatment of the pseudopeptide **50b** with TFA for 2 h gave a 5:1 mixture of two products, one of which (minor) was the expected triacid containing a P−OMe ester, while the other was the fully hydrolyzed tetraacid **52**. Reaction of the mixture with TFA overnight led to complete hydrolysis of compound **50b** to give **52** (Scheme 8). This result indicates that the phosphinic acid methyl ester is as sensitive to acidic hydrolysis as are the carboxylic acid *tert*-butyl esters. Tetraacid **52** was then used in an attempt to prepare the corresponding tetra-POM ester **53** using POMI under the standard conditions. However, the yield of **53** was very low (ca 10%), in addition to a mixture of partially esterified mono-, di-, and tri-POM esters.[42]

Scheme 9



Given the inability to convert free tetraacid **52** to the desired POM ester precursor, **53**, a strategy was adopted to install the POM ester on the precursors prior to the formation of either C−P bond of the phosphinate pseudopeptide. It was found that POMI is stable to overnight exposure (room temperature) to aqueous $Na_2S_2O_3$ solution (Y. Feng, unpublished results). This suggested that perhaps the POM ester moiety of a phosphinate pseudopeptide would survive exposure to the numerous aqueous-organic extractions in a multistep synthesis if all P−C bonds were synthesized under acidic or neutral conditions after installation of the POM esters on the N-terminal and C-terminal fragments. This approach was then investigated (Scheme 9). Initially, methyl 4-bromobutyrate **41** was converted to phenylselenide **54**, which, following hydrolysis to the free acid **55**, was esterified with POMI to give POM ester **56** in excellent overall yield. It should be noted that the successful use of alkyl bromide **41** as the electrophilic partner in the synthesis of selenide **54** is in marked contrast to the failure of **41** to undergo reaction with two nucleophilic P^{III} species, derived from **35** and **42**, in the attempted synthesis of **1b** (Scheme 5) and **45** (Scheme 6), respectively. Evidence for the attenuated nucleophilicity of P^{III} species in reactions with unactivated alkyl halides has been reported previously.[16]

Neutral conditions were chosen to carry out the oxidative elimination of the phenylselenide moiety to afford the corresponding olefin.[62] The POM ester **56** was heated at reflux temperature in a mixture of H_2O_2 and THF. As expected, the oxidative elimination reaction proceeded very smoothly and the desired *N*-Cbz-vinylglycine POM ester **57** was obtained in excellent yield. This result showed that under these conditions, the POM ester is stable. Encouraged by this result, **57** was subsequently converted to the H-phosphinic acid **58** using Montchamp's phosphorus radical reaction.[16,49] Michael addition of **58** to di-POM-α-methyleneglutarate (**34**) was accomplished by BSA-mediated, in situ generation of the nucleophilic bis-TMS derivative of **58** and monitoring of the reaction by ^{31}P NMR.[16] The use of $TMSCHN_2$ in a mixture of toluene and methanol to trap the resulting phosphinic acid as its corresponding methyl ester **59** resulted in complete methanolysis of the POM ester. However, use of CH_2N_2 in EtOAc resulted in formation of the desired tri-POM ester **59** in 46% yield over three steps. Removal of the *N*-Cbz group of tris-POM pseudopeptide **59** afforded the secondary amine **60**. Reaction of **60** with **11b** (generated in situ from **11a**) led to a mixture of POM ester **1b** and the corresponding free phosphinic acid, **1c**, the latter presumably arising from halide-mediated demethylation (Figure 2). Methylation of the mixture with CH_2N_2 in EtOAc led to POM ester **1b**, in modest yield. Since HPLC data showed that the substitution reaction between **60** and **11b**, followed by treatment with CH_2N_2, proceeded with complete consumption of **60** to yield a mixture of **1b** and triphenylphosphine oxide, the modest yield of the last step may be due to the lability of the POM ester to nucleophiles such as H_2O and MeOH under conditions used for purification by preparative HPLC or preparative TLC.

Table 2. Initial Cytotoxicity Data for Prodrug Forms of FPGS Substrates and Inhibitors (Chinese hamster ovary cells;74 IC_{50}, nM)

compd	Pro-3	R2	43-10	compd	Pro-3	R2	43-10
1a	>1000	>1000	>1000	**2a**	5	350	5
1b	>1000	>1000	800	**2b**	8	350	9
1c	700	350	250	**2c**	20	350	3
1d	500	>1000	300	**2d**	6	75	5

Biological Data and Hydrolytic Stability. Cytotoxicity assays were carried out in collaboration with Prof. Larry Matherly, Karamanos Cancer Center and Department of Pharmacology, Wayne State University, Detroit, MI. Five prodrug esters (**1b**-**1d**, **2b**, **2d**) of the phosphinic acid-containing pseudopeptide **1a**, methotrexate **2a**, and its γ-glutamyl "conjugate" **2c**, were evaluated in three cell lines derived from Chinese hamster ovaries (CHO). These included wild-type cells Pro3, with a fully functional reduced folate carrier (RFC);[38] a mutant cell line, R2, which is ca. 70-fold less sensitive to antifolates due to a marked decreased expression of RFC;[38] and the cell line 43−10, in which sensitivity to antifolates has been restored as a result of transfection of RFC cDNA to R2 cells, thereby restoring high sensitivity to MTX.[39] As shown in Table 2, pseudopeptides **1a**−**d** were uniformly ineffective in all cell lines. However, the prodrug forms of MTX (AMPteGlu) and its γ-glutamyl conjugate, AMPteGlu-γGlu, were effective and

Table 3. Half-Lives of the Prodrugs in Phosphate Buffer and Cell Medium[a]

compd	$t_{1/2}$ (h) phosphate buffer (20 mM, pH 8.0)	$t_{1/2}$ (h) cell medium[b] (pH 8.0)
1b	3	1
1c	8	3
1d	>100	—[c]
2b	3.5	2
2d	0.8	0.5

[a] Determined by HPLC. [b] RPMI-1640 medium. [c] Not determined.

in several cases equipotent with the parent compound, i.e., **2b** vs **2a**, **2d** vs **2c**. As noted in the Introduction, the POM esters of MTX and its γ-glutamyl conjugate were synthesized for use as controls. It was anticipated that the prodrug forms **2b** and **2d** would cross the cell membrane by diffusion, after which the esters would be hydrolyzed to provide MTX or AMPteGlu-γ-Glu, respectively.

These data suggest that hydrolysis of the prodrug esters is occurring, either via an enzyme-catalyzed or nonenzymic route, prior to and/or after entering the cell. If true, the resulting γ-glutamyl peptide derived from **2d** would be a substrate for γ-glutamyl hydrolase (GH, EC 3.4.19.9), a secreted cysteine peptidase,[37] leading to free MTX (**2a**). However, the free acid-containing phosphinic acid pseudopeptide **1a**, the desired hydrolysis product of **1b**, **1c**, and **1d**, does not penetrate the cell membrane[15] and cannot be a GH substrate. Thus, extracellular hydrolysis of all esters moieties of **1b**−**1d**, **2b**, and **2d** would lead, respectively, to a highly polar phosphapeptide **1a**, which is unable to cross the cell membrane or to free MTX (**2a**), the latter either directly from **2b** or via GH-catalyzed hydrolysis of the isopeptide bond of **2c** following ester hydrolysis (**2d** → **2c** → **2a**).[63] The proposed extracellular hydrolysis would explain the cytotoxicity data, i.e., the small effect of the POM ester moieties on cell penetration and cytotoxicity of phosphinic acid pseudopeptides **1b**−**d** vs toxicity of POM esters **2b** and **2d**, which suggest ultimate hydrolysis to MTX (**2a**). Similar results were obtained in vivo with **2b** vs **2a** and have been reported in the patent literature.[64] It should be noted that the free phosphinic acids, **1c** and **1d**, have considerably improved solubility when compared to the P−OMe ester **1b**. These solubility differences may explain the marginal improvement in cytotoxicity noted for **1c** and **1d** vs **1b**.

A search of the literature revealed a paucity of kinetics data on the hydrolysis of POM esters in aqueous buffer or cell medium. Therefore, we undertook a study of the stability of the four prodrug esters in phosphate buffer (20 mM, pH 8.0) and also in cell medium (RPMI-1640, pH 8.0). A comparison of prodrug half-lives determined in phosphate buffer and cell culture medium is given in Table 3. As expected on the basis of literature precedent,[65] POM esters **1b**, **1c**, **2b**, and **2d** were more labile than the methyl ester **1d**. The more rapid reaction of prodrug esters in cell medium vs phosphate buffer is presumably due to the presence in medium of numerous oxygen, nitrogen, and sulfur nucleophiles (e.g., amino acids, glutathione, vitamins, etc.) in addition to water.

The wide variety of nucleophilic reactions other than hydrolysis that are possible in cell medium led us to focus a more detailed analysis of the hydrolytic stability of prodrug esters **1b**−**d**, **2b**, and **2d** in phosphate buffer, pH 8.0, as described below. The bis-POM ester of MTX, i.e., AMPteGlu(α,γ-$OPOM_2$), **2b**, was the first compound studied in detail (Figure 3). Product analysis (LC−MS) revealed that a product containing only one POM ester was formed much more rapidly than a

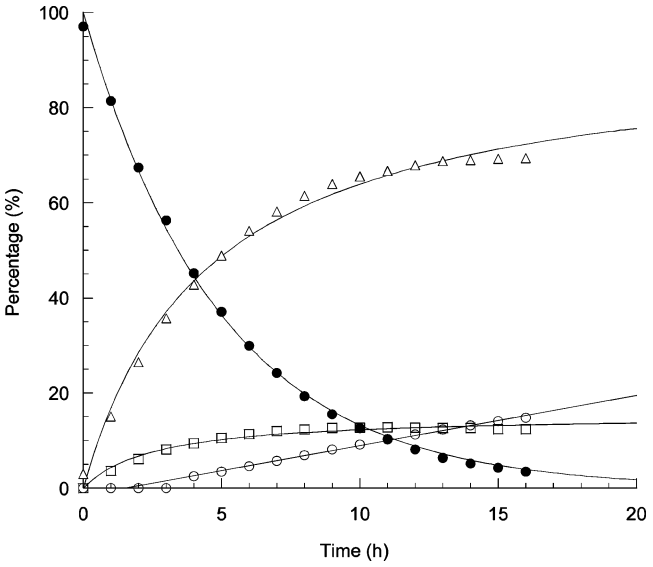
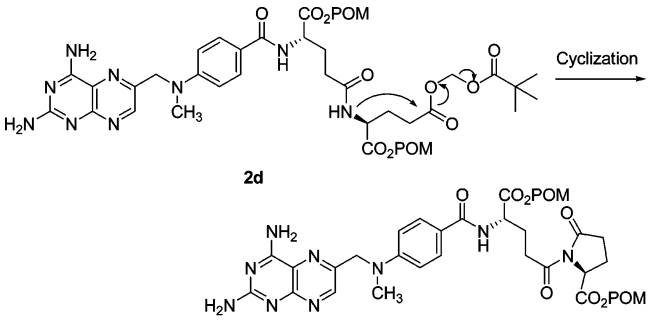


Figure 3. Hydrolysis of AMPteGluPOM_2 (**2b**) in phosphate buffer (20 mM, pH 8.0, 5% DMSO). Data for the hydrolysis of **2b** (●) are fit to a pseudo-first-order kinetics equation. Formation of the mono-POM derivatives (△, □) is fit to a rectangular hyperbola. Following a lag period of ca. 3 h, formation of MTX (**2a**) (○) occurs with a linear dependence on time.

second mono-POM derivative, which in turn was formed slightly faster than the completely hydrolyzed product, MTX (**2a**). The overall hydrolysis scheme can be summarized as follows: AMPteGlu(α,γ-$OPOM_2$) (**2b**) → AMPteGlu(α- or γ-OPOM) → AMPteGlu(α- or γ-OPOM) → AMPteGlu (**2a**). On the basis of steric considerations, we hypothesize that the γ-POM ester of **2b** is more labile than the α-POM ester, thus leading to initial rapid hydrolysis of the γ-POM ester and slower hydrolysis of the α-POM ester. Formation of the free acid MTX (**2a**) is observed, albeit at a rate indicating that hydrolysis of the initially formed major product is slow. After prolonged reaction (115 h), the observed products were a mono-POM ester (ca. 60%), presumed to be AMPteGlu(α-OPOM-γ-OH), and **2a** (ca. 40%), with none of the initially formed minor product, presumed to be AMPteGlu(α-OH-γ-OPOM), remaining.

Hydrolysis of AMPteGlu(α-OPOM)-γ-Glu(α,γ-$OPOM_2$) (**2d**) proceeded much more rapidly ($t_{1/2}$ = ca. 0.5 h) than was observed with **2b** (data not shown). However, it was soon determined that the product of this reaction was not the corresponding free acid but rather a pyroglutamic acid derivative **61**, resulting from intramolecular reaction of the peptide nitrogen on the γ-POM ester moiety (Scheme 10). Subsequent hydrolysis of each of the remaining two α-POM esters occurred with $t_{1/2}$ = ca. 4 h in phosphate buffer, thus providing an estimate for

Scheme 10



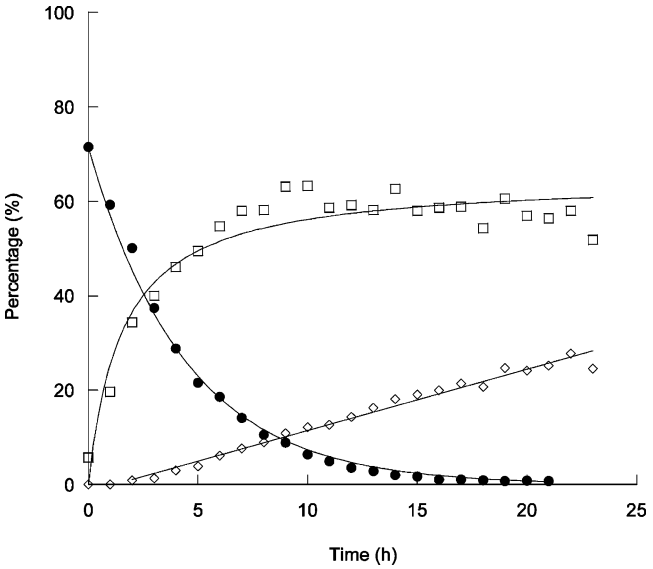


Figure 4. Hydrolysis of AMPteGlu[P(O)(OMe)]GluPOM$_3$ (**1b**) in Phosphate buffer (20 mM, pH 8.0, 5% DMSO). Data for the hydrolysis of **1b** (●) are fit to pseudo-first-order kinetics. Formation of the bis-POM derivative(s) (□) is fit to a rectangular hyperbola. Following a lag period of ca. 3 h, formation of the mono-POM derivatives (◇) occurs with linear dependence on time. At any time point, the sum of peak percentage data is not 100% due to the formation of several unidentified minor products during these experiments as monitored by HPLC.

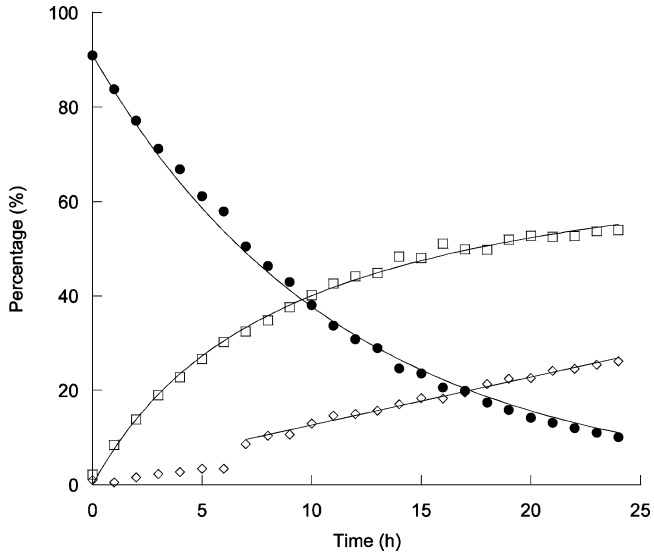
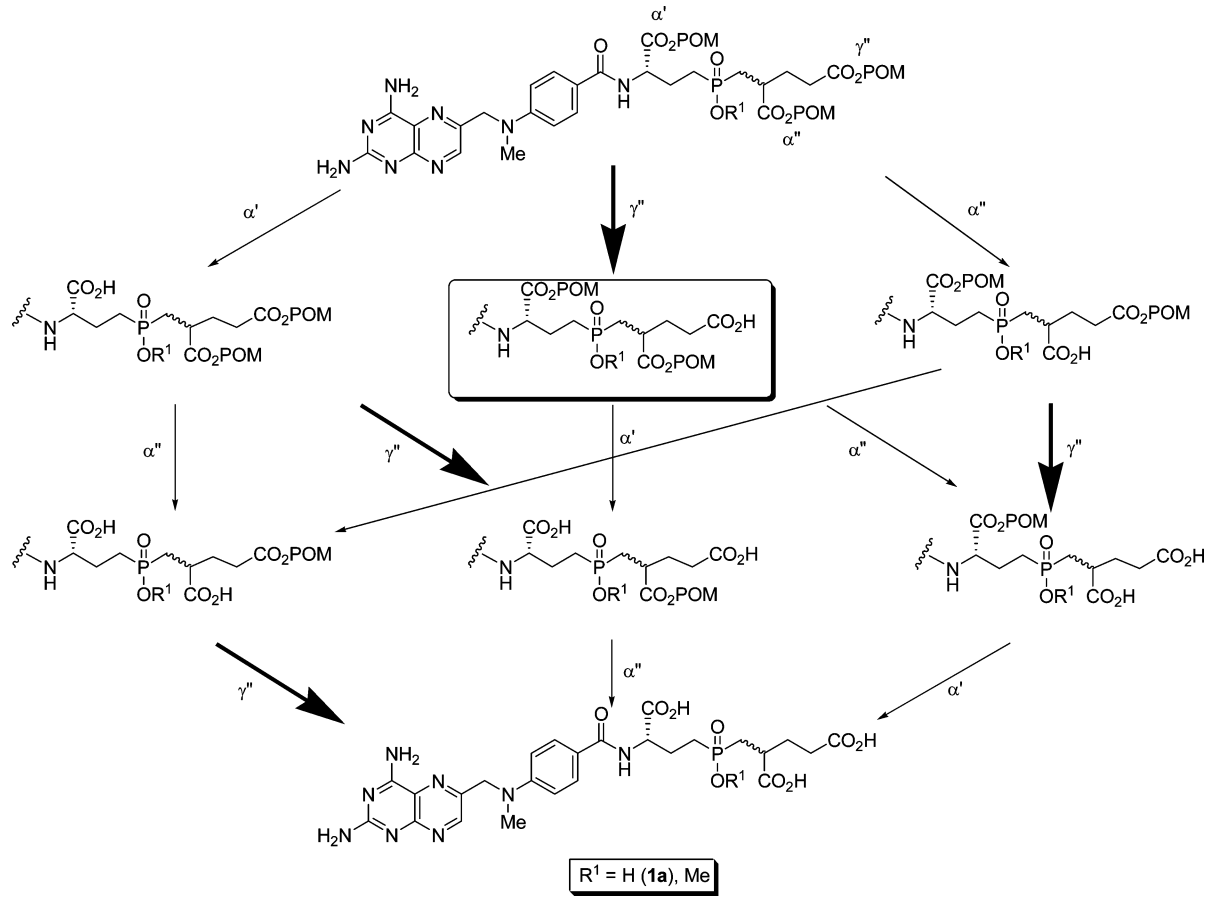


Figure 5. Hydrolysis of AMPteGlu[P(O)(OH)]GluPOM$_3$ (**1c**) in phosphate buffer (20 mM, pH 8.0, 5% DMSO). Data for the hydrolysis of **1c** (●) are fit to pseudo-first-order kinetics. Formation of the bis-POM derivative(s) (□) is fit to a rectangular hyperbola. Following a lag period of ca. 6 h, formation of the mono-POM derivatives (◇) occurs with linear dependences on time. At any time point, the sum of peak percentage data is not 100% due to the formation of several unidentified minor products during these experiments as monitored by HPLC.

the rate of hydrolysis of α-POM esters in related compounds of interest in this research.

Hydrolysis of phosphapeptides **1b**−**1d** each proceeded at a very different rate with half-lives of 3 h (**1b**, Figure 4), 8 h (**1c**, Figure 5), and >100 h (**1d**, data not shown). These results are consistent with the literature reports that the POM esters are more susceptible to hydrolysis than the corresponding methyl esters.[65] LC−MS data indicate that the tris-POM derivatives,

Scheme 11



1b and **1c**, react initially to form a mixture of bis-POM derivatives, followed by a much slower loss of POM esters to produce the free acid, **1a**, via a series of mono-POM derivatives. The difference in hydrolysis rates of bis-POM species derived from the P−OMe (**1b**) and P−OH (**1c**) derivatives presumably reflects the fact that the α-esters are closer to the central phosphinic ester or acid, respectively, than the initially hydrolyzed γ-ester. The phosphinic acid **1c** is ionized at pH 8.0 and the more proximal α-esters may be less prone to attack by negatively charged nucleophiles such as hydroxide ion. After a more extended reaction time (24 h), the distribution of the bis-POM:mono-POM derivatives of both **1b** and **1c** was determined to be ca. 2:1. These are thought to be the γ-COOH, bis-α-POM ester derivative and a mixture of two γ-CO_2H, mono-α-POM esters, derived from precursors containing free γ-CO_2H and free α-CO_2H, respectively, assuming that hydrolysis of the γ-ester is faster than the α-ester as hypothesized above.

The postulated hydrolysis pathways are shown in Scheme 11, in which hydrolysis at the γ-ester, indicated in bold, leads ultimately to one bis-POM ester and two mono- POM esters. Only small amounts of the final product, a triacid (R^1 = Me) from **1b** or tetraacid (**1a**, R^1 = H) from **1c**, were observed under these conditions. All routes to these final products involve a combination of hydrolytic reactions at one γ-ester and two α-esters, with the latter being rate-limiting and, in the event, preventing significant formation of **1a** or its P−OMe ester.

The research described in this paper (Figures 4 and 5, Scheme 11) provides the first data on the hydrolytic lability of isopeptide prodrug esters in phosphate buffer and cell medium, pH 8.0. The results obtained with **1b**−**d** indicate that spontaneous hydrolysis of the prodrug esters does not lead to the parent compounds, **1a**. In addition, the high lipophilicity of POM esters **1b** (log P = 3.50), **1c** (log P = 2.21), **2b** (log P = 2.54), and **2d** (log P = 2.90), results in solubility problems that have been noted by others.[42,66] Thus, hydrolytic stability combined with poor aqueous solubility leads to poor bioavailability of this initial series of pseudopeptide prodrugs. Studies on the lability of these esters in cell lysates and with cloned esterases are underway, and the results will be reported in due course. Future research will focus on the synthesis and evaluation of compounds with alternate prodrug esters and/or containing a free γ-carboxyl group, both of which should result in compounds with better solubility properties as well as improved bioavailability.

Conclusion

Several routes to synthesize prodrug esters of methotrexate, its γ-glutamyl conjugate, and a phosphinate pseudopeptide were investigated. The most effective method for synthesis of a pseudopeptide POM ester prodrug uses N-Cbz vinylglycine POM ester as the key intermediate to incorporate the N-terminal C−P bond and (α,γ-bis-POM)-α-methyleneglutarate to set the C-terminal P−C bond. Several other approaches proved to be impractical for a variety of reasons. POM esters were found to be quite stable under neutral or mild acidic conditions but more labile under basic conditions. However, hydrolysis of the phosphonic pseudopeptide tetramethyl ester was ineffective under common basic conditions, whereas while the phosphinic acid P−OMe moiety was sensitive to acid, such as TFA, and also to halide ion. Conditions required to effect phosphorus−carbon bond formation were also investigated and optimized. Prodrug esters **1b**, **1d**, and **2b** and **2d** were synthesized successfully by coupling of 2,4-diamino-6-(bromomethyl)-pteridine with the *N*-*p*-ABA derivatives of peptide or pseudopeptide esters. Moreover, the synthetic route to the tris-POM ester **1b** of phosphinate pseudopeptide **1a** allows for the installation of varied ester moieties on different carboxylic acids, to give additional prodrug candidates for structure−activity relationship (SAR) investigations.

All newly synthesized pseudopeptide (**1**) and peptide (**2**) prodrugs were evaluated in three cell lines derived from Chinese hamster ovaries (CHO), including wild-type cells with a fully functional reduced folate carrier (RFC), a mutant cell line with decreased expression of RFC, and a cell line in which RFC function has been restored by transfection of RFC-deficient CHO cells with human RFC cDNA. Although the pseudopeptide prodrugs **1b**−**d** were ineffective in all cell lines, prodrugs **2b** and **2d** were effective and in several cases equipotent with their parent compounds, MTX and its γ-glutamyl conjugate, **2a** and **2c**, respectively. The cytotoxicity data suggested that hydrolysis of the prodrugs esters may have occurred prior to entering the cell. Thus, the hydrolysis rate of the prodrugs was investigated in both phosphate buffer (pH 8.0) and cell line medium (pH 8.0). In phosphate buffer, all of the prodrugs, except the tris-methyl ester **1d**, could be hydrolyzed with the loss of a single POM moiety within a few hours. In cell line medium, the initial hydrolysis rates of the prodrugs were even faster. The results indicate that POM ester prodrugs of substituted glutamic acid (e.g., MTX and its γ-glutamyl conjugates, or phosphinate pseudopeptides) contain one hydrolytically sensitive POM ester moiety, presumably at the more accessible C-terminal γ-carboxyl group. Hydrolysis of the remaining POM esters, presumably at the α-carboxyl group(s), is much slower. The latter esters may be substrates for cellular esterases, a hypothesis that is currently being tested experimentally. Since the C-terminal γ-POM ester is assumed to be the most labile, its removal in future prodrug candidates may be appropriate. The desired lipophilicity of prodrug esters results in poor aqueous solubility, a characteristic that could be partially ameliorated by the synthesis of prodrug esters of γ-glutamyl peptides or pseudopeptides containing a free C-terminal γ-carboxylic acid. Research on the development of this and other second-generation prodrugs of **1a** is currently underway in our laboratory.

Experimental Section

General Procedures. All reactions involving moisture-sensitive reagents were carried out under an inert atmosphere of nitrogen or argon in oven-dried glassware. All solvents used in moisture-sensitive reactions were dried as follows. Tetrahydrofuran and toluene were freshly distilled over sodium/benzophenone. Dichloromethane was freshly distilled over CaH_2. Triethylamine was distilled from KOH and stored over 4 Å molecular sieves. Thin-layer chromatography was performed with aluminum-backed silica gel 60-F254 plates. Column chromatography was performed with silica gel 60 (230−400 mesh) according to the protocol of Still et al.[67] When applicable, column chromatography was carried out by using the CombiFlash system (ISCO, Inc.) for better separation. Melting points were taken on a Thomas-Hoover Mel-Temp apparatus and are uncorrected. All NMR spectra were recorded on a Bruker AVANCE DRX300 300-MHz or AVANCE DRX500 500-MHz spectrometer. ^{1}H NMR spectra were recorded and are reported as follows: chemical shifts in ppm downfield from internal tetramethylsilane with multiplicity, integrated intensity, coupling constant in hertz. ^{13}C NMR spectra were obtained at 75 or 126 MHz and referenced to tetramethylsilane. Assignment of the ^{1}H and ^{13}C peaks was aided by acquisition of DEPT and 2D spectra (COSY, HETCOR) of the compounds. Unless otherwise mentioned, the reagents used were purchased from Aldrich or Fisher. Ammonium hypophosphite was purchased from Fluka and dried over P_2O_5 under vacuum for 2 days at room temperature prior to use. Analytical HPLC method A: flow rate, 1 mL/min; eluant A, phosphate buffer (20 mM, pH 7.0); eluant B, acetonitrile; gradient,

0 min, 2% B, 35 min, 50% B, 90 min, 50% B; column, Chrompack Kromasil 100 C18, 250 × 4.6 mm. Analytical HPLC method B: flow rate, 1 mL/min; eluant A, ammonium acetate buffer (20 mM, pH 6.5); eluant B, acetonitrile; gradient, 0 min, 2% B, 30 min, 80% B, 40 min, 80% B; column, Chrompack Kromasil 100 C18, 250 × 4.6 mm). Detection was by UV/vis, $\lambda = 254$ and 310 nm. Pivaloyloxymethyl iodide (POMI) was prepared according to the literature,[43] decolorized with saturated aqueous NaS_2O_3 solution, and purified by distillation (bp 64−66 °C/10−11 mmHg, lit.[43] bp 35 °C/0.1 mmHg). *N*-(*N*-Cbz-L-glutamyl-γ)-L-glutamic acid,[44] as well as compounds **11a**,[51,68,69] **22**,[70] **24a**,[71,72] **24b**,[71,73] **40**,[16,50] and **46a**,[16] were prepared according to the literature procedure. Experimental details on the synthesis of **46b** from **40**, via oxidative elimination of a selenide, are given in the Supporting Information, since such details and spectral data are not available in the literature.[60,61]

Bis(pivaloyloxymethyl) *N*-benzyloxycarbonyl-L-glutamate (5). To a solution of *N*-Cbz-L-glutamic acid (281 mg, 1 mmol) in 5 mL of DMF was added silver carbonate (828 mg, 3 mmol), dimethyl methylphosphonate (620 mg, 5 mmol), and POMI (1.21 g, 5 mmol). The color of the resulting mixture changed from gray greenish to yellowish slowly. The resulting mixture was kept at room temperature overnight and the precipitate was moved by filtration. The residue was washed with EtOAc. Some precipitate was formed in the filtrate again. The filtering operation was repeated twice until the filtrate was clear. The filtrate was then combined, washed with saturated $NaHCO_3$ and brine, and dried over $NaSO_4$. The solvent was removed to give a yellow oil as the crude product, which was purified by flash chromatography (EtOAc:hexanes = 1:5, $R_f = 0.4$) to yield 409 mg (80%) of **5** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.32−7.42 (m, 5H), 5.74−5.91 (m, 4H), 5.45 (s, br, 1H), 5.12−5.21 (m, 2H), 4.47 (s, br, 1H), 2.49−2.54 (m, 2H), 2.25−2.27 (m, 1H), 2.01−2.06 (m, 1H), 1.20−1.32 (m, 18H). ^{13}C NMR ($CDCl_3$): 177.46, 177.26, 171.62, 171.01, 156.35, 136.46, 129.10, 128.92, 128.78, 128.62, 128.51, 80.32, 80.04, 67.53, 53.48, 39.01, 38.80, 30.17, 27.43, 27.28, 27.21, 27.18. ESI-HRMS (*m*/*z*): calcd for $C_{25}H_{35}NO_{10}Na$ $[M + Na]^+$ 532.2159, found 532.2161.

Bis(pivaloyloxymethyl) *N*-[*N*-benzyloxycarbonyl-α-pivaloyloxymethyl-L-glutamyl-γ]-L-glutamate (6). To a solution of the *N*-(*N*-Cbz-L-glutamyl-γ)-L-glutamic acid (100 mg, 0.36 mmol) in 5 mL of DMF was added dimethyl methylphosphonate (446 mg, 3.6 mmol) and Ag_2CO_3 (607 mg, 2.2 mmol) followed by the addition of POMI (871 mg, 3.6 mmol). Some gas was released. The color of the suspension changed from gray to yellow. The mixture was then stirred under room temperature for 18 h. The precipitate was filtered off, and the filtrates were poured into 200 mL of EtOAc. The whole mixture was washed with saturated $NaHCO_3$ and brine and dried over Na_2SO_4. Removal of the solvent gave a yellow oil as the crude product, which was purified with flash chromatography (EtOAc:hexanes = 1:2, $R_f = 0.55$) to give 126 mg (70%) of **6** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.32 (s, 5H), 6.60 (d, J = 7.34 Hz, 1H), 5.59−5.88 (m, 6H), 5.03 (s, 2H), 4.54−4.61 (m, 1H), 4.33−4.35 (m, 1H), 2.39−2.43 (m, 2H), 2.26−2.31 (m, 2H), 2.15−2.16 (m, 2H), 1.95−2.00 (m, 2H), 1.18 (s, 27H). ^{13}C NMR ($CDCl_3$): δ 177.55, 177.48, 177.29, 172.29, 171.73, 171.18, 170.83, 156.55, 136.54, 128.90, 128.57, 128.49, 80.35, 80.25, 80.11, 67.46, 53.76, 51.88, 46.42, 39.12, 32.13, 30.26, 27.44. ESI-HRMS (*m*/*z*): calcd for $C_{36}H_{52}N_2O_{15}Na$ $[M + Na]^+$ 775.3265, found 775.3278.

Pivaloyloxymethyl L-pyroglutamate (9a) and Pivaloyloxymethyl *N*-methyl-L-pyroglutamate (9b). To a solution of di-POM *N*-Cbz-glutamate (**6**) (102 mg, 0.2 mmol) in 10 mL of EtOH was added 20 mg of Pd/C. The resulting mixture was then stirred under H_2 at room temperature overnight. Removal of the Pd/C and solvent gave 61 mg of the crude product, which was purified by flash chromatography (EtOAc:hexanes = 3:1, $R_f = 0.4$) to give 22 mg (45%) of **9a** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 6.63 (s, 1H), δ 5.84 (d, J = 5.5 Hz, 1H), 5.80 (d, J = 5.5 Hz, 1H), 4.30 (dd, J_1 = 5.0 Hz, J_2 = 8.7 Hz, 1H), 2.41−2.51 (m, 1H), 2.34−2.39 (m, 2H), 2.20−2.24 (m, 1H), 1.22 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.48, 171.42, 161.23, 80.11, 49.70, 38.81, 27.23, 21.32, 21.14. Further elution (EtOAc:hexanes = 3:1, $R_f = 0.2$) to give 21 mg (40%) of **9b** as a colorless oil. ^{1}H NMR ($CDCl_3$): 5.85 (d, J = 5.5 Hz, 1H), 5.79 (d, J = 5.5 Hz, 1H), 4.10−4.17 (m, 1H), 2.85 (s, 3H), 2.34−2.44 (m, 3H), 2.05−2.06 (m, 1H), 1.21 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.42, 171.45, 161.34, 80.23, 49.82, 38.81, 30.25, 27.23, 21.45, 21.01.

Di-*tert*-butyl *N*-[4-(*N*-benzyloxycarbonyl-*N*-methyl)aminobenzoyl]-L-glutamate (25a). Diphenylphosphoryl azide (1.375 g, 5 mmol) and triethylamine (505 mg, 5 mmol) were added into a solution of acid **22** (1.14 g, 4 mmol) in 5 mL of DMF. The solution was stirred at room temperature for 2.5 h. Di-*tert*-butyl L-glutamate **24a** (1.554 g, 6 mmol) and another portion of triethylamine (606 mg, 6 mmol) were added. The resulting reaction mixture was then stirred at room temperature overnight. The resulting turbid mixture was poured into 200 mL of EtOAc and washed with 1 N HCl, $NaHCO_3$, and brine. After being dried over Na_2SO_4, the solvent was removed on a rotary evaporator to give a yellow oil as the crude product, which was purified by flash chromatography (EtOAc: hexanes = 1:1, $R_f = 0.6$) to give 2.1 g (90% over two steps) of **25a** as an oil. ^{1}H NMR ($CDCl_3$): δ 7.81 (d, J = 8.52 Hz, 2H), 7.33 (m, 5H), 7.08 (d, J = 7.34 Hz, 1H), 5.18 (s, 2H), 4.66−4.68 (m, 1H), 3.35 (s, 3H), 2.20−2.44 (m, 3H), 2.04−2.09 (m, 1H), 1.41−1.50 (m, 18H). ^{13}C NMR ($CDCl_3$): 173.04, 171.69, 166.69, 155.45, 146.57, 136.65, 131.36, 128.92, 128.51, 128.32, 128.17, 125.40, 82.82, 81.28, 68.02, 53.30, 37.80, 32.05, 28.45, 28.42, 27.81. ESI-HRMS (*m*/*z*): calcd for $C_{29}H_{38}N_2O_7Na$ $[M + Na]^+$ 549.2577, found 549.2592.

α-*tert*-butyl *N*-[4-(*N*-benzyloxycarbonyl-*N*-methyl)aminobenzoyl]-L-glutamate (25b). Diphenylphosphoryl azide (206 mg, 0.75 mmol) and triethylamine (101 mg, 1 mmol) were added into a solution of acid **22** (75 mg, 0.5 mmol) in 2 mL of DMF. The solution was stirred for 2.5 h. α-*tert*-butyl glutamate **24b** (102 mg, 0.5 mmol) and tetramethylguanidine (138 mg, 1.2 mmol) were added and dissolved in 5 min. Two days later, the solvent was removed. The residue was acidified with 5% citric acid and extracted with EtOAc. The extracts were washed with H_2O and brine and dried over Na_2SO_4. Purification of the crude product with silica gel column chromatography (methanol:CH_2Cl_2 = 1:15, R_f = 0.3) gave 146 mg (86%) of the desired product as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 10.30 (s, br, 1H), 7.80 (d, J = 8.61 Hz, 2H), 7.28−7.38 (m, 7H), 7.11 (d, J = 7.50 Hz, 1H)), 5.30 (s, 2H), 4.68−4.75 (m, 1H), 3.33 (s, 3H), 2.45−2.53 (m, 2H), 2.27−2,32 (m, 1H), 2.03−2.10 (m, 1H), 2.17 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.75, 171.59, 167.66, 155.52, 146.69, 136.55, 131.00, 128.95, 128.57, 128.36, 128.24, 125.41, 83.32, 68.12, 53.05, 37.77, 30.69, 28.40, 28.03. ESI-HRMS (*m*/*z*): calcd for $C_{25}H_{30}N_2O_7Na$ $[M + Na]^+$ 493.1951, found 492.1954.

Di-*tert*-butyl *N*-{α-*tert*-butyl-*N*-[4-(*N*-benzyloxycarbonyl-*N*-methylamino)benzoyl]-L-glutamyl-γ}-L-glutamate (26) Diphenylphosphoryl azide (330 mg, 1.2 mmol) and triethylamine (151 mg, 1.5 mmol) were added into a solution of acid **25b** (470 mg, 1 mmol) in 3 mL of DMF. The solution was stirred for 2.5 h. Di-*tert*-butyl L-glutamate (102 mg, 0.5 mmol) and tetramethylguanidine (173 mg, 1.5 mmol) were added. The turbid resulting mixture was then kept at room temperature overnight. The resulting mixture was poured into 200 mL of EtOAc and washed with 2 N HCl (40 mL × 3), $NaHCO_3$ (40 mL ×3), and brine (40 mL x 3). After being dried over Na_2SO_4, the solvent was removed on a rotary evaporator to give a colorless oil as the crude product, which was purified by flash chromatography (EtOAc:hexanes = 1:1, $R_f = 0.35$) to yield 628 mg (86%) of the title compound was obtained as an oil. ^{1}H NMR ($CDCl_3$): δ 7.83 (d, J = 8.55 Hz, 2H), 7.54 (d, J = 6.81 Hz, 1H), 7.28−7.34 (m, 7H), 6.65 (d, J = 7.32 Hz, 1H). 5.17 (s, 2H), 4.57−4.62 (m, 1H), 4.45−4.48 (m, 1H), 3.33 (s, 3H), 2.25−2.36 (m, 4H), 2.03−2.10 (m, 4H), 0.90−1.51 (m, 27H). ^{13}C NMR ($CDCl_3$): δ 172.65, 172.61, 171.57, 171.38, 166.91, 155.44, 146.50, 136.64, 131.27, 128.91, 128.48, 128.29, 125.38, 82.72, 82.66, 81.10, 67.98, 60.78, 53.50, 52.76, 37.80, 32.83, 31.88, 28.44, 28.39, 28.34, 21.44, 14.59. ESI-HRMS (*m*/*z*): calcd for $C_{38}H_{53}N_3O_{10}Na$ $[M + Na]^+$ 734.3629, found 734.3649.

Bis(pivaloyloxymethyl) *N*-[4-(*N*-benzyloxycarbonyl-*N*-methyl)-aminobenzoyl]-L-glutamate (27). A solution of the di-*tert*-butyl ester **25a** (2.1 g, 4 mmol) in 5 mL of dichloromethane was cooled to 0 °C and TFA (5 mL) was added dropwise. The resulting mixture was then warmed to room temperature and stirred overnight. The solvent was removed on a rotary evaporator to give a sticky oil, which was put on the pump overnight. The oil was then dissolved in 20 mL of DMF. To the solution was added dimethyl methylphosphonate (7.44 g, 30 mmol), silver carbonate (8.28 g, 30 mmol), and POMI (14.5 g, 30 mmol). The whole was then stirred at room temperature overnight. The solid was filtered off. The filtrate was collected and poured into 400 mL of EtOAc. The combined organic solution was washed with $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed on a rotary evaporator to give a black yellow oil, which was purified by silica gel column chromatography (EtOAc:hexanes = 1:2, $R_f = 0.3$) to yield 1.5 g (60%) of the desired product as a pale yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.81 (d, $J = 8.70$ Hz, 2H) 7.26−7.40 (m, 7H), 6.95 (d, $J = 7.45$, 1H), 5.91 (d, $J = 5.49$ Hz, 1H), 5.71−5.80 (m, 3H), 5.20 (s, 2H), 4.82−4.83 (m, 1H), 3.38 (s, 3H), 2.52−2.58(m, 2H), 2.16 (m, 1H), 2.14−2.16 (m, 1H), 1.20−1.23 (m, 18H). ^{13}C NMR ($CDCl_3$): 177.48, 177.33, 172.26, 171.01, 166.90, 155.43, 146.86, 136.61, 130.71, 128.94, 128.55, 128.34, 128.22, 125.43, 80.45, 80.10, 68.08, 52.52, 39.16, 39.12, 37.75, 30.49, 27.23, 26.96. ESI-HRMS (*m*/*z*): calcd for $C_{33}H_{42}N_2O_{11}Na$ $[M + Na]^+$ 665.2686, found 665.2695.

Bis(pivaloyloxymethyl) *N*-{α-pivaloyloxymethyl-*N*-[4-(*N*-Cbz-*N*-methyl)aminobenzoyl]-L-glutamyl-γ}-L-glutamate (28). To a solution of the *tert*-butyl ester **26** (711 mg, 1 mmol) in 5 mL of CH_2Cl_2 at 0 °C was added 5 mL of TFA dropwise. The resulting solution was warmed to room temperature and stirred for another 4 h. The solvent was removed in vacuo. The TFA in the residue was removed by multiple coevaporations with CH_2Cl_2 and hexanes. The resulting residue was then put on the high-vacuum pump overnight to afford 560 mg (quantitative) of **27** as a white oily solid. ^{1}H NMR (MeOH-d_4): δ 7.88 (d, $J = 8.60$ Hz, 2H), 7.40 (d, $J = 8.60$ Hz, 2H), 7.29−7.35 (m, 5H), 5.16 (s, 2H), 4.57−4.60 (m, 1H), 4.43−4.46 (m, 1H), 3.34 (s, 3H), 2.44−2.47 (m, 2H), 2.38−2.41 (m, 2H), 2.30−2.34 (m, 1H), 2.10−2.18 (m, 2H), 1.90−1.93 (m, 1H). ^{13}C NMR (MeOH-d_4): δ 175.3, 174.2, 174.0, 173.9, 168.6, 155.9, 146.6, 136.7, 131.6, 128.6, 128.3, 128.2, 128.0, 125.5, 67.8, 53.0, 52.1, 36.8, 32.1, 30.2, 27.0, 26.9, 26.7.

To a solution of acid **27** prepared above (163 mg, 0.3 mmol) in 15 mL of DMF were added dimethyl methylphosphonate (1.12 g, 9 mmol), Ag_2CO_3 (745 mg, 2.7 mmol), and POMI (2.18 g, 9 mmol). The resulting mixture was allowed to stir at room temperature overnight. The solution was then concentrated and the residue was extracted with EtOAc. The extracts were combined and washed with saturated $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed to give 346 mg of a red oil as the crude product, which was purified by flash chromatography (EtOAc: hexanes = 1:1, $R_f = 0.3$) to yield 227 mg (86%) of **28** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.83 (d, $J = 8.62$ Hz, 2H), 7.71 (d, $J = 6.66$ Hz, 1H), 7.29−7.36 (m, 7H), 6.65 (d, $J = 7.60$ Hz, 1H), 5.90 (d, $J = 5.47$ Hz, 1H), 5.78 (d, $J = 5.47$ Hz, 1H), 5.75 (d, $J = 2.05$ Hz, 1H), 5.74 (d, $J = 2.05$ Hz, 1H), 5.70 (d, $J = 5.49$ Hz, 1H), 5.66 (d, $J = 5.49$ Hz, 1H), 4.67−4.71 (m, 1H), 4.60−4.64 (m, 1H), 2.39−2.51 (m, 4H), 2.24−2.30 (m, 1H), 2.13−2.23 (m, 2H), 2.00−2.06 (m, 1H), 1.21 (s, 9H), 1.20 (s, 9H), 1.19 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.6, 177.5, 177.3, 173.0, 171.8, 171.0, 170.6, 167.0, 155.4, 146.8, 136.7, 128.9, 128.5, 128.3, 125.4, 80.5, 80.4, 80.2, 68.0, 53.1, 52.1, 39.2, 39.1, 37.8, 32.4, 30.3, 27.5, 27.3, 27.2, 27.0, 26.9, 21.4. ESI-HRMS (*m*/*z*): calcd for $C_{44}H_{59}N_3O_{16}Na$ $[M + Na]^+$ 908.3793, found 908.3816.

Bis(pivaloyloxymethyl) *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate (29). The di-POM ester **27** (642 mg, 1 mmol) was dissolved in 10 mL of ethanol. To the solution was added 130 mg (20%) of 5% palladium on activated carbon. The mixture was saturated with hydrogen and stirred overnight under H_2, after which the catalyst was removed by filtration with a short silica pad. TLC showed the product was sufficiently pure for use in the next step without further purification. Evaporation of solvent gave 480 mg (95%) of **29**. ^{1}H NMR ($CDCl_3$): δ 7.64 (d, $J = 8.61$ Hz, 2H), 6.77 (d, $J = 7.47$ Hz, 1H), 6.54 (d, $J = 8.64$ Hz, 2H), 5.86 (d, $J = 5.43$, 1H), 5.67−5.74 (m, 3H), 4.76−4.83 (m, 1H), 4.33 (s, br, 1H), 2.84 (s, 3H), 2.46−2.58 (m, 2H), 2.25−2.31 (m, 1H), 2.02−2.14 (m, 1H), 1.16−1.20 (m, 18H). ^{13}C NMR ($CDCl_3$): δ 177.52, 177.36, 172.24, 171.42, 167.54, 152.70, 129.28, 121.32, 111.69, 80.30, 80.00, 52.24, 39.13, 39.10, 30.56, 30.50, 27.21. ESI-HRMS (*m*/*z*): calcd for $C_{25}H_{36}N_2O_9Na$ $[M + Na]^+$ 531.2319, found 531.2328.

Bis(pivaloyloxymethyl) *N*-[(4-deoxy-2,4-diamino-10-methyl)-pteroyl]-L-glutamate (2b). To a suspension of dibromotriphenylphosphine (253 mg, 0.6 mmol) in 1 mL of dimethylacetamide was added (2,4-diamino-6-pteridinyl)methanol hydrochloride (**11a**) (45 mg, 0.2 mmol). The resulting mixture turned to clear and red in a few minutes with emission of gas. The reddish solution was stirred at room temperature for 18 h before diethylisopropylamine (103 mg, 0.8 mmol) and a solution of the POM ester **29** (152 mg, 0.3 mmol) in dimethylacetamide (1 mL) was added. The resulting reaction mixture was then kept at 60 °C for 2 days. The solvent was removed and the residue was extracted with 200 mL of EtOAc. The extracts were combined and washed with $NaHCO_3$ and brine and dried over sodium sulfate. Removal of the solvent gave 302 mg of the crude product, which was purified by silica gel column chromatography (MeOH:CH_2Cl_2 = 1:20, $R_f = 0.3$) to yield 113 mg (83%) of **2b** as a yellow oil. ^{1}H NMR (methanol-d_4): δ 8.49 (s, 1H), 7.68 (d, $J = 8.80$ Hz, 2H), 6.76 (d, $J = 8.86$ Hz, 2H), 5.68−5.82 (m, 4H), 4.75 (s, 2H), 4.58 (dd, J_1=9.14 Hz, J_2=5.46 Hz, 1H), 3.28−3.29 (m, 1H), 3.17 (s, 3H), 2.50 (t, $J = 7.28$, 2H), 2.03−2.24 (m, 2H), 1.11 (s, 18H). ^{13}C NMR (methanol-d_4): δ 177.32, 177.24, 171.90, 171.18, 169.26, 163.64, 162.99, 154.45, 152.15, 149.21, 148.16, 129.29, 122.33, 121.00, 111.58, 80.07, 79.81, 55.54, 52.47, 38.71, 30.13, 26.24, 25.87; IR (KBr, cm^{-1}): 3332, 3161, 2975, 1755, 1632, 1607, 1557, 1509, 1480, 1450, 1366, 1332, 1282, 1201, 1157, 1114, 1032, 986, 827, 767, 736. ESI-MS (*m*/*z*): $[M + Na]^+$: 705.3. HRMS (*m*/*z*): calcd for $C_{32}H_{42}N_8O_9$ $[M + Na]^+$ 705.2972, found 705.2967. UV λ_{max}: (0.1 N NaOH) 255, 306, 378, 460 nm; (0.1 N HCl) 245, 308. Analytical HPLC method A: $t_R = 47.6$ min. Analytical HPLC method B: 27.4. Anal. ($C_{32}H_{42}N_8O_9$·0.5H_2O): C, H, N.

Bis(pivaloyloxymethyl) *N*-{α-pivaloyloxymethyl-*N*-[4-(*N*-methyl)aminobenzoyl]-L-glutamyl-γ}-glutamate (30). To a solution of the POM ester **28** (204 mg, 0.23 mmol) in 10 mL of EtOH was added 40 mg of Pd/C, and the mixture stirred under H_2 at room temperature overnight, after which the catalyst was removed by filtration. Evaporation of the filtrate gave 180 mg of the crude product, which was purified by silica gel column chromatography (EtOAc:hexanes = 1:1, $R_f = 0.25$) to yield 165 mg (96%) of **30** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.68 (d, $J = 8.5$ Hz, 2H), 7.23 (d, $J = 6.8$ Hz, 1H), 6.81 (d, $J = 7.5$ Hz, 1H), 6.59 (d, $J = 8.5$ Hz, 2H), 5.88 (d, $J = 5.5$ Hz, 1H), 5.78 (d, $J = 5.5$ Hz, 1H), 5.74 (d, $J = 3.4$ Hz, 1H), 5.73 (d, $J = 3.4$ Hz, 1H), 5.70 (d, $J = 5.5$ Hz, 1H), 5.67 (d, $J = 5.5$ Hz, 1H), 4.69−4.73 (m, 1H), 4.58−4.63 (m, 1H), 2.35−2.52 (m, 4H), 2.17−2.28 (m, 2H), 2.06−2.14 (m, 1H), 1.98−2.05 (m, 1H), 1.21 (s, 9H), 1.20 (s, 9H), 1.19 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.6, 177.5, 177.3, 173.0, 171.7, 171.4, 170.7, 167.7, 152.4, 129.4, 121.7, 112.0, 80.4, 80.3, 80.1, 52.8, 52.0, 39.2, 39.1, 32.6, 30.8, 30.3, 27.7, 27.6, 27.2, 26.9. ESI-HRMS (*m*/*z*): calcd for $C_{36}H_{53}N_3O_{14}Na$ $[M + Na]^+$ 774.3425, found 774.3445.

Bis(pivaloyloxymethyl) *N*-{*N*-[(4-deoxy-2,4-diamino-10-methyl)pteroyl]-α-pivaloyloxymethyl-L-glutamyl-γ}-glutamate (2d). To the suspension of dibromotriphenylphosphine (152 mg, 0.36 mmol) in 1 mL of dimethylacetamide (DMA) was added (2,4-diamino-6-pteridinyl)methanol hydrochloride (**11a**) (28 mg, 0.12 mmol). The resulting mixture turned to clear and red in a few minutes with emission of gas. The reddish solution was stirred at room temperature for 20 h before diisopropylethylamine (62 mg, 0.48 mmol) and a solution of the POM ester **30** (135 mg, 0.18 mmol) in dimethylacetamide (1 mL) was added. The resulting mixture was then kept at 60 °C for 30 h and monitored by analytical HPLC. The solvent was removed and the residue was extracted

with 200 mL of EtOAc. The extracts were combined and washed with $NaHCO_3$ and brine and dried over Na_2SO_4. Removal of the solvent gave 310 mg of the crude product, which was purified by silica gel column chromatography (MeOH:CH_2Cl_2 = 1:20, R_f = 0.25) to yield 83 mg (75%) of **2d** as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 8.56 (s, 1H), 7.65 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 5.9 Hz, 1H), 7.43 (d, J = 7.2 Hz, 1H), 6.89 (s, br, 2H), 6.67 (d, J = 8.4 Hz, 2H), 6.25 (s, br, 2H), 5.86 (d, J = 5.5 Hz, 1H), 5.77 (d, J = 5.5 Hz, 1H), 5.72 (d, J = 4.8 Hz, 1H), 5.71 (d, J = 4.8 Hz, 1H), 5.68 (d, J = 5.4 Hz, 1H), 5.64 (d, J = 5.4 Hz, 1H), 4.60−4.66 (m, 4H), 3.11 (s, 3H), 2.40−2.49 (m, 4H), 2.20−2.24 (m, 1H), 2.13−2.19 (m, 2H), 1.99−2.05 (m, 1H), 1.19 (s, 18H), 1.17 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.5, 177.4, 177.3, 173.2, 171.7, 171.6, 171.0, 167.7, 163.4, 163.0, 155.4, 151.9, 149.9, 147.4, 129.4, 122.4, 121.5, 111.8, 80.4, 80.3, 80.1, 56.1, 52.9, 52.0, 39.5, 39.1, 39.1, 32.5, 30.3, 27.5, 27.2, 27.1, 26.8; IR (KBr, cm^{-1}):3322, 3155, 2974, 2935, 2874, 1755, 1607, 1558, 1535, 1508, 1481, 1449, 1368, 1332, 1281, 1200, 1157, 1112, 1029, 985, 942, 854, 827, 766. ESI-MS (*m*/*z*): $[M + Na]^+$: 948.4. HRMS (*m*/*z*): calcd for $C_{43}H_{59}N_9O_{14}$ $[M + Na]^+$ 948.4079, found 948.4086. UV λ_{max} (phosphate buffer, pH 7): 225, 360, 310, 380 nm^{-1}. Analytical HPLC method A: t_R = 58.2 min. Analytical HPLC method B: t_R = 29.9 min. Anal. ($C_{43}H_{59}N_9O_{14}$•0.5H_2O): C, H, N.

Dimethyl α-methyleneglutarate (32a). Methyl acrylate (**31a**) (45 mL, 43 g, 500 mmol) was cooled to −10 °C, followed by the addition of tri-*n*-butylphosphine (10.1 g, 50 mmol). (**Caution:** *This reaction is exothermic*). The reaction was allowed to warm to room temperature, and after stirring for 1.5 h, the solution was concentrated on a rotary evaporator and the residue distilled under reduced pressure to give 27.9 g (65%) of **32a** as a colorless oil (bp 95−98 °C/10−11 Torr, lit.[52] bp 74−77 °C/9.5 Torr). ^{1}H NMR ($CDCl_3$): δ 6.20 (s, 1H), 5.61 (s, 1H), 3.76 (s, 3H), 3.64 (s, 3H), 2.64 (t, J = 6.57 Hz, 2H), 2.52 (t, J = 7.86 Hz). ^{13}C NMR ($CDCl_3$): δ 173.52, 167.51, 139.17, 126.38, 52.31, 52.01, 33.30, 27.73.

Di-*tert*-butyl α-methyleneglutarate (32b). To neat *tert*-butyl acrylate (**31b**) (4.38 g, 34 mmol) was added *tri*-*n*-butylphosphine (808 mg, 4 mmol) at room temperature, and the resulting mixture was stirred for 2 h. The desired product was then isolated through distillation (bp 95−98 °C/2 mmHg, lit.[74] bp 67−70 °C/0.3 mmHg) to afford 3.27 g (75%) of **32b** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 6.08 (s, 1H), 5.49 (d, J = 1.27 Hz, 1H), 2.55 (t, J = 7.39 Hz, 2H), 2.55 (t, J = 7.5 Hz, 2H), 2.41 (t, J = 7.5 Hz, 2H), 1.54 (s, 9H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 172.59, 166.43, 141.12, 124.78, 81.06, 80.73, 34.74, 28.50, 28.46, 27.96.

α-Methyleneglutaric acid (33). A mixture of the dimethyl ester **32a** (3.44 g, 20 mmol), NaOH (2.4 g, 60 mmol), 10 mL of methanol, and 10 mL of H_2O was heated to reflux for 2 h. The solution was acidified with 6 N HCl to pH < 1. The methanol was removed on a rotary evaporator. The aqueous mixture was then extracted with EtOAc (100 mL × 5). The organic extracts were combined and dried over Na_2SO_4. Removal of the solvent afforded 2.76 g (96%) of a white solid as the desired product. Mp: 130−132 °C (Lit.[16] mp: 130−136 °C). ^{1}H NMR (DMSO-d_6): δ 12.33 (s, 1H), 6.04 (s, 1H), 5.59 (s, 1H), 2.34∼2.50 (m, 4H). ^{13}C NMR (DMSO- d_6): δ 174.55, 168.63, 140.50, 125.67, 33.34, 27.61.

Bis(pivaloyloxymethyl) α-methyleneglutarate (34). To the solution of the acid **33** (710 mg, 5 mmol) in 10 mL of DMF were added Ag_2CO_3 (4.14 g, 15 mmol), dimethyl methylphosphonate (3.72 g, 30 mmol), and POMI (7.26 g, 30 mmol). The resulting mixture was stirred overnight. The solid was filtered off. The residue was washed with 200 mL of EtOAc. The filtrate was collected and washed with $NaHCO_3$ and brine. After being dried over Na_2SO_4, the solvent was removed. Purification of the residue by silica gel column chromatography (EtOAc:hexanes = 1:8, R_f = 0.55) gave 780 mg (42%) of **34** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 6.27 (s, 1H), 5.82 (s, 2H), 5.73 (s, 2H), 5.70 (s, 1H), 2.64 (t, J = 6.87 Hz, 2H), 2.55 (t, J = 6.16 Hz, 2H), 1.19 (s, 18H). ^{13}C NMR ($CDCl_3$): δ 177.51, 171.57, 165.39, 137.93, 128.40, 80.12, 79.81, 39.17, 39.11, 32.96, 27.22. ESI-HRMS (*m*/*z*): calcd for $C_{18}H_{28}O_8$Na $[M + Na]^+$ 395.1682, found 395.1683.

Bis(pivaloyloxymethyl) 2-[(hydroxyphosphinoyl)methyl]glutarate (35). The suspension of ammonium hypophosphite (249 mg, 3 mmol) and 1,1,1,3,3,3-hexmethyldisilazane (497 mg, 3.1 mmol) was heated at ∼110 °C under argon. Some gas was released and some sublimate was formed on the wall of the flask. Ninety minutes later, the mixture turned homogeneous and then cooled to room temperature, followed by the addition of the solution of the POM ester **34** (186 mg, 0.5 mmol) in 2 mL of freshly distilled CH_2Cl_2. The resulting mixture was stirred at room temperature overnight. HCl (5 mL, 2 N) was used to quench the reaction. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (30 mL × 3). The organic extracts were combined and dried over Na_2SO_4. Removal of the solvent gave 205 mg (94%) of **34** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 9.99 (s, 1 H), 7.15 (d, J = 567.4 Hz, 1H), 5.69−5.84 (m, 4H), 2.90 (m, 1H), 2.37−2.43 (m, 2H), 2.17∼2.20 (m, 1H), 1.89−2.03 (m, 3H), 1.15−1.22 (m, 18H). ^{13}C NMR ($CDCl_3$): δ 177.44, 177.32, 172.73, 172.64, 171.40, 80.20, 79.96, 39.10, 38.17, 31.77, 31.16, 30.51, 27.40, 27.21. ^{31}P NMR ($CDCl_3$): δ 35.02. ESI-HRMS (*m*/*z*): calcd for $C_{18}H_{31}O_{10}$PNa $[M + Na]^+$ 461.1553, found 461.1554.

(2*S*)-2-*N*-[4-(*N*-carbobenzyloxy-*N*-methyl)aminobenzoyl]aminobutyrolactone (36). To a solution of acid **22** (1.14 g, 4 mmol) were added diphenylphosphoryl azide (1.1 g, 4 mmol) and triethylamine (505 mg, 5 mmol). The resulting solution was stirred at room temperature for 2.5 h, followed by the addition of (*S*)-2-aminobutyrolactone hydrobromide **20** (728 mg, 4 mmol). The resulting mixture was allowed to react at room temperature overnight and then poured into 200 mL of EtOAc, washed with saturated $NaHCO_3$ and brine and dried over Na_2SO_4. Removal of the solvent and purifying the crude product with silica gel column chromatography gave 1.4 g (95%) of **36** as a white solid. ^{1}H NMR ($CDCl_3$): δ 7.76 (d, J = 8.60 Hz 2H), 7.23−7.30 (m, 7H), 7.21 (d, J = 6.40 Hz, 1H) 5.15 (s, 2H), 4.74 (q, J = 8.74 Hz, 1H), 4.37 (t, J = 8.79 Hz, 1H), 4.19 (dd, J_1=8.90 Hz, J_2=6.51 Hz, 1H), 3.28 (s, 3H), 2.59 (m, 1H), 2.31 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 176.40, 176.24, 167.34, 167.27, 155.48, 146.69, 136.57, 130.39, 128.94, 128.55, 128.38, 128.25, 125.24, 68.04, 66.53, 60.82, 49.70, 37.67, 29.57, 21.45, 14.59. ESI-HRMS (*m*/*z*): calcd for $C_{20}H_{20}N_2O_5$Na $[M + Na]^+$ 391.1270, found 391.1273.

(2*S*)-2-*N*-[4-(*N*-carbobenzyloxy-*N*-methyl)aminobenzoyl]amino-4-hydroxybutyric Acid (37). To a solution of the lactone **36** (736 mg, 2 mmol) in 3 mL of methanol was added 3 mL of 1 N NaOH. The mixture was stirred at room temperature for 2 h. The methanol was removed on a rotary evaporator. The resulting aqueous mixture was acidified with 2 N HCl and extracted with EtOAc. The extracts were put together and dried over dried over Na_2SO_4. Removal of the solvent gave 710 mg (92%) of **37** as a colorless oil, which was used without further purification. ^{1}H NMR (CD_3OD): δ 7.85 (d, J = 7.76 Hz, 2H), 7.36 (d, J = 8.18 Hz, 2H), 7.30 (m, 5H), 5.14 (s, 2H), 4.80 (t, J = 9.72 Hz, 1H), 4.44 (t, J = 8.22 Hz, 1H), 4.29 (q, J = 7.28 Hz, 1H), 3.28 (s, 3H), 2.53 (m, 1H), 2.41 (t, J = 10.4 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 176.61, 168.02, 155.72, 146.76, 136.73, 130.83, 128.67, 128.30, 128.24, 125.40, 67.84, 66.40, 49.53, 36.90, 28.47, 20.00. ESI-HRMS (*m*/*z*): calcd for $C_{20}H_{22}N_2O_6$Na $[M + Na]^+$ 409.1376, found 409.1362.

(2*S*)-Methyl 4-bromo-2-*N*-[4-(*N*-carbobenzyloxy-*N*-methyl)-aminobenzoyl]aminobutyrate (41). To a solution of the free acid **40** (395 mg, 1.5 mmol) in 5 mL of anhydrous MeOH at 0 °C was added $SOCl_2$ (0.22 mL, 3 mmol) dropwise. The resulting solution was then warmed to room temperature and stirred overnight. Removal of the solvent in a vacuum gave 783 mg of a yellow solid as the desired product, which was used in the next step without purification. To the mixture of the product obtained above and *p*-(*N*-carbobenzoyloxy-*N*-methyl-amino)benzoic acid (456 mg, 1.6 mmol) in 10 mL of freshly distilled CH_2Cl_2 at 0 °C were added Et_3N (162 mg, 1.6 mmol) and EDC (307 mg, 1.6 mmol). The solution was then warmed to room temperature and stirred for 2 h. HCl (2 mL, 2 N) was added to quench to reaction. The resulting mixture was diluted with 100 mL of EtOAc, washed with $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent gave 603 mg of the crude product, which was purified by flash chroma-

tography (hexanes:EtOAc = 1:1, R_f = 0.5) to give 528 mg (76% over two steps) of **41** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.77 (d, J = 8.54 Hz, 2H), 7.25−7.29 (m, 7H), 5.14, (s, 2H), 3.79 (s, 3H), 3.58 (t, J = 6.56 Hz, 1H), 3.42 (t, J = 6.82 Hz, 1H), 3.28 (s, 3H), 2.29−2.49 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 172.60, 167.20, 155.44, 146.64, 136.58, 130.86, 128.92, 128.54, 128.38, 128.25, 125.27, 68.03, 52.89, 52.16, 41.34, 37.70, 35.35, 29.17. ESI-HRMS (*m*/*z*): calcd for $C_{21}H_{23}BrN_2O_5Na$ [M + Na]$^+$ 485.0688, found 485.0683.

Dimethyl *N*-[(*N*-carbobenzyloxy-α-methyl)-L-glutamyl-γ]-[Ψ-{P(O)(OCH$_3$)CH$_2$}]-glutarate (48a). To a solution of the methyl vinylglycine **46a** (360 mg, 1.44 mmol) in 10 mL of methanol were added ammonium hypophosphite (300 mg, 3.6 mmol) and triethylborane (1 M in hexanes, 1.5 mL, 1.5 mmol). The resulting mixture was stirred open to the air at room temperature for 2 h. The solvent was then removed on a rotary evaporator while the temperature of the water bath was kept lower that 30 °C. $KHSO_4$ (20 mL, 1 M) was added to the residue. The resulting aqueous mixture was then extracted with EtOAc (50 mL × 3). The organic extracts were combined and dried over Na_2SO_4. Removal of the solvent gave 430 mg of the desired phosphinic acid **47a**, which was used in the next step without purification. ^{1}H NMR (methanol-d_4): δ 6.69 (d, J = 546 Hz, 1H), 7.00 (m, 5H), 4.76 (s, 2H), 3.94−3.96 (m, 1H), 3.38 (s, 3H), 1.68−1.77 (m, 1H), 1.46−1.58 (m, 3H). ^{13}C NMR (methanol-d_4): δ 170.91, 155.85, 135.38, 126.84, 126.41, 126.18, 65.09, 52.91, 52.68, 50.29, 21.62. ^{31}P NMR (CD_3OD): δ 33.36.

A solution of the phosphonic acid **47a** (505 mg, 1.6 mmol) obtained above and dimethyl α-methyleneglutarate (550 mg, 3.2 mmol) in 10 mL of fresh-distilled CH_2Cl_2 was dried over $MgSO_4$ and then injected into a round-bottom flask through a syringe filter under argon. BSA (1.02 g, 5 mmol) was added. The resulting solution was stirred at room temperature overnight. The resulting mixture was allowed to open to the air, followed by addition of 5 mL of 2 N HCl. The mixture was stirred vigorously for 2 h. The organic layer was separated. The aqueous layer was extracted with EtOAc (10 mL × 3). The organic solutions were then combined and dried over Na_2SO_4. Removal of the solvent gave 1.2 g of a colorless oil as the crude product, which was used in the next step without further purification.

The product obtained above was dissolved in 15 mL of MeOH, followed by addition of trimethylsilyldiazomethane (2.0 M in Et_2O, 2 mL, 4 mmol) until the color of the mixture remained green yellow. The resulting mixture was then stirred at room temperature for another hour. A drop of AcOH was added, resulting in loss of the color. Removal of the solvent gave 1.3 mg of a yellow oil as the crude product, which was purified with CombiFlash (MeOH:EtOAc = 1:50, R_f = 0.2) to yield 600 mg (75% over 3 steps) of **48a** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.89−7.92 (m, 2H), [7.78 (d, J = 6.8 Hz, 0.5H), 7.73 (d, J = 6.9 Hz, 0.2H), 7.69 (d, J = 7.0 Hz, 0.3H), amide N−H, 3 rotamers], 7.29−7.36 (m, 7H), 4.76−4.82 (m, 1H), 3.78−3.79 (m, 3H), 3.61−3.73 (m, 9H), 2.80−2.86 (m, 1H), 2.33−2.37 (m, 2H), 2.27−2.32 (m, 2H), 2.24−2.27 (m, 1H), 1.93−2.00 (m, 3H), 1.75−1.87 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 174.9, 174.8, 173.2, 173.1, 172.5, 172.4, 167.0, 155.4, 146.7, 136.7, 130.9, 128.9, 128.5, 128.4, 128.3, 125.4, 68.0, 53.04, 53.02, 52.62, 52.60, 52.2, 52.0, 51.92, 51.87, 51.7, 39.1, 38.9, 37.8, 31.54, 31.45, 29.2, 24.5. ^{31}P NMR ($CDCl_3$): δ 57.42, 57.36, 56.8. ESI-HRMS (*m*/*z*): calcd for $C_{22}H_{32}NO_{10}PNa$ [M + Na]$^+$ 524.1662, found 524.1666.

Di-*tert*-butyl (α-*tert*-butyl-*N*-carbobenzyloxy-L-glutamyl-γ)-[Ψ{P(O)(OMe)CH$_2$}]-glutarate (48b). To a solution of the *tert*-butyl vinylglycine **46b** (122 mg, 0.4 mmol) in 2 mL of methanol were added ammonium hypophosphite (91 mg, 1.1 mmol) and triethylborane (1 M in hexanes, 0.5 mL, 0.5 mmol). The resulting mixture was stirred open to the air at room temperature for 3 h. The solvent was then removed on a rotary evaporator while the temperature of the water bath was kept lower than 30 °C. To the residue was added 10 mL of 1 M $KHSO_4$. The resulting aqueous mixture was then extracted with EtOAc (20 mL × 3). The organic extracts were combined and dried over Na_2SO_4. Removal of the solvent gave 150 mg of the desired product **47b**, which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$): δ 11.18 (s, br, 1H), 7.29−7.34 (m, 5H), 7.07 (d, J = 553 Hz, 1H), 5.09 (s, 2H), 4.29 (m, 1H), 2.03−2.11 (m, 1H), 1.78−1.90 (m, 3H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 170.98, 156.58, 136.55, 128.94, 128.62, 128.54, 83.26, 67.52, 54.81, 30.09, 28.34, 24.39. ^{31}P NMR ($CDCl_3$): δ 37.61. MS (*m*/*z*, M + Na$^+$): 380.

To the solution of the H-phosphinic acid obtained above (290 mg, 0.8 mmol) was added the solution of di-*tert*-butyl α-methyleneglutarate (614 mg, 2.4 mmol) in 2 mL of freshly distilled CH_2Cl_2 dried over $MgSO_4$ through a syringe with a filter under argon, followed by addition of BSA (812 mg, 4 mmol). The resulting solution was heated at reflux temperature overnight and then quenched with 2 N HCl (5 mL). The mixture was stirred vigorously for 3 h. The organic layer was separated. The aqueous layer was extracted with EtOAc (10 mL × 3). The organic solutions were then combined and dried over Na_2SO_4. Removal of the solvent gave 970 mg of the crude product as a colorless oil, which was used in the next step without further purification.

The product obtained above was dissolved in 5 mL of MeOH, followed by addition of trimethylsilyldiazomethane (2.0 M in Et_2O, 1 mL, 2 mmol) until the color of the mixture remained green yellow. The resulting mixture was then stirred at room temperature for another hour. A drop of AcOH was added to quench the reaction. Removal of the solvent gave 986 mg of the crude product, which was purified by flash chromatography (EtOAc:hexanes = 3:2, R_f = 0.3) to yield 209 mg (42% over three steps) of **48b** as a colorless oil.^{1}H NMR ($CDCl_3$): δ 7.29−7.35 (m, 5H), 5.60−5.67 (m, 1H), 5.09 (s, 2H), 4.25−4.26 (m, 1H), 3.62−3.73 (m, 3H), 2.67 (m, 1H), 2.22−2.26 (m, 2H), 2.16−2.19 (m, 2H), 1.86−1.89 (m, 3H), 1.71−1.74 (m, 3H), 1.45 (s, 9H), 1.44 (s, 9H), 1.43 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 173.77, 172.30, 172.26, 172.23, 171.01, 156.40, 136.67, 128.89, 128.53, 83.00, 82.96, 82.94, 81.80, 81.74, 81.01, 80.96, 67.34, 54.94, 51.62, 51.42, 39.79, 39.59, 33.02, 32.96, 30.54, 30.07, 29.81, 29.72, 29.36, 28.46, 28.38, 28.36, 25.51, 25.36, 25.11, 24.74, 24.48, 24.38, 24.01. ^{31}P NMR ($CDCl_3$): δ 57.23, 57.12, 56.68, 56.62. ESI-HRMS (*m*/*z*): calcd for $C_{31}H_{50}NO_{10}PNa$ [M + Na]$^+$ 650.3070, found 650.3075.

Dimethyl (α-methyl-L-glutamyl-γ)-[Ψ{P(O)(OMe)CH$_2$}]-glutarate (49a). To a solution of the tetramethyl phosphinic pseudopeptide **48a** (501 mg, 1 mmol) in 20 mL of freshly distilled THF was added 100 mg of 5% Pd/C, and the mixture was saturated with H_2. The resulting mixture was then stirred at room temperature under H_2 overnight. The Pd/C was filtered off. The filtrate was collected and concentrated to give 370 mg of an oil as the crude product, which was purified by flash chromatography (MeOH:EtOAc = 1:4, R_f = 0.4) to yield 348 mg (95%) of **49a** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 3.74 (s, 3H), 3.72 (s, 3H), 3.68 (s, 3H), 3.64−3.71 (m, 3H), 3.48−3.51 (m, 1H), 2.82−2.88 (m, 1H), 2.33−2.38 (m, 2H), 2.22−2.26 (m, 1H), 1.97−2.03 (m, 3H), 1.77−1.88 (m, 4H), 1.68 (s, br, 2H). ^{13}C NMR ($CDCl_3$): δ 175.93, 175.04, 175.00, 173.32, 173.28, 54.89, 54.77, 52.58, 52.55, 52.15, 51.67, 51.61, 51.56, 51.51, 39.02, 38.83, 38.80, 31.66, 31.56, 30.71, 30.43, 29.99, 29.72, 29.23, 29.14, 27.36, 25.48, 25.14, 24.83, 24.75, 24.40. ^{31}P NMR ($CDCl_3$): δ 56.97, 56.56. ESI-HRMS (*m*/*z*): calcd for $C_{14}H_{26}NO_8PNa$ [M + Na]$^+$ 390.1294, found 390.1289.

Di-*tert*-butyl (α-*tert*-butyl-L-glutamyl-γ)-[Ψ{P(O)(OMe)CH$_2$}]-glutarate (49b). This compound was obtained in 95% yield from **48b** using the same procedure described above for **49a**. ^{1}H NMR ($CDCl_3$): δ 4.05 (m, 1H), 3.68−3.77 (m, 3H), 2.66−2.72 (m, 1H), 2.37 (m, 2H), 2.18−2.28 (m, 4H), 2.11−2.13 (m, 1H), 1.84−1.91 (m, 3H), 1.51 (s, 9H), 1.46 (s, 9H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 173.74, 173.68, 173.63, 172.40, 172.37, 172.35, 168.19, 84.58, 84.55, 81.76, 81.71, 81.63, 80.94, 80.92, 53.87, 52.50, 52.45, 52.34, 52.29, 52.20, 52.14, 52.09, 39.78, 39.50, 33.08, 33.04, 32.99, 30.53, 30.35, 30.09, 29.71, 29.60, 28.49, 28.42, 28.38, 28.20, 24.84, 24.38, 24.13, 23.69, 23.52. ^{31}P NMR ($CDCl_3$): δ 57.74, 57.35. ESI-HRMS (*m*/*z*): calcd for $C_{37}H_{57}N_2O_{15}PNa$ [M + Na]$^+$ 494.2883, found 494.2888.

Dimethyl {*N*-[*p*-(*N*-carbobenzyloxy-*N*-methylamino)benzoyl]-α-methyl-L-glutamyl-γ}-[Ψ{P(O)(OMe)CH$_2$}]-glutarate (50a). A solution of the free amine **49a** (300 mg, 0.82 mmol) and *p*-(*N*-

carbobenzyloxy-*N*-methylamino)benzoic acid (285 mg, 1 mmol) in 10 mL of fresh-distilled CH_2Cl_2 was stirred at 0 °C under N_2 for 5 min, followed by addition of EDCl (192 mg, 1 mmol) in one portion. The resulting mixture was then warmed to room temperature and stirred for 2 h. The reaction mixture was put in an ice−water bath and 2 mL of 2 N HCl was added to quench the reaction. The resulting mixture was poured into 200 mL of EtOAc, washed with $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent gave 610 mg of the crude product, which was purified by silica gel column chromatography (2% MeOH in EtOAc, $R_f = 0.4$) to yield 478 mg (92%) of **50a** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.89−7.92 (m, 2H), 7.78 [(d, J = 6.84 Hz, 0.5 H), 7.73 (d, J = 6.89 Hz, 0.2 H), 7.69 (d, J = 7.00 Hz, 0.3 H), amide N−H, three rotamers], 7.28−7.36 (m, 7H), 3.78−3.79 (m, 3H), 3.61−3.75 (m, 9H), 2.80−2.86 (m, 1H), 2.33−2.37 (m, 2H), 2.24−2.32 (m, 2H), 2.10−2.22 (m, 1H), 1.93−2.00 (m, 3H), 1.75−1.87 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 174.88, 174.84, 173.22, 173.18, 172.48, 172.43, 166.97, 155.45, 146.69, 136.66, 130.90, 128.92, 128.51, 128.44, 128.32, 125.40, 68.02, 53.04, 53.02, 52.62, 52.57, 52.16, 51.92, 51.87, 51.74, 39.12, 38.88, 37.78, 31.54, 31.45, 29.29, 24.54. ^{31}P NMR ($CDCl_3$): δ 57.42, 57.36, 56.84. ESI-HRMS (*m*/*z*): calcd for $C_{30}H_{39}N_2O_{11}PNa$ [M + Na]$^+$ 657.2189, found 657.2204.

Di-*tert*-butyl {*N*-{*p*-[(*N*-carbobenzyloxy-*N*-methyl)amino]benzoyl}-α-*tert*-butyl-L-glutamyl-γ}-[Ψ{P(O)(OMe)CH$_2$}]-glutarate (50b). This compound was obtained in 92% yield from **49b** using the same procedure described above for **50a**. ^{1}H NMR ($CDCl_3$): δ 7.88 (d, J = 7.87 Hz), 7.46−7.53 (m, 1H), 7.29−7.35 (m, 7H), 5.18 (s, 2H), 4.64−4.71 (m, 1H), 3.60−3.71 (m, 3H), 3.34 (s, 3H), 2.66 (m, 1H), 2.18−2.24 (m, 4H), 2.09−2.14 (m, 1H), 1.80−1.99 (m, 3H), 1.69−1.78 (m, 2H), 1.49 (s, 9H), 1.42 (s, 9H), 1.41−1.46 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 173.73, 172.24, 172.22, 172.19, 172.17, 171.17, 171.13, 166.91, 166.83, 155.44, 146.57, 136.66, 131.22, 128.91, 128.48, 128.32, 128.29, 125.40, 82.98, 82.96, 82.94, 81.80, 81.77, 81.75, 80.95, 67.99, 53.88, 53.84, 53.78, 53.74, 53.68, 53.59, 51.74, 51.69, 51.55, 51.50, 39.89, 39.82, 39.68, 39.65, 37.79, 33.00, 32.96, 32.91, 30.69, 30.30, 30.23, 29.96, 29.80, 29.70, 29.58, 29.51, 28.46, 28.41, 28.37, 25.21, 25.01, 24.94, 24.78, 24.49, 24.11, 24.05. ^{31}P NMR ($CDCl_3$): δ 57.84, 57.72, 57.35, 57.26. ESI-HRMS (*m*/*z*): calcd for $C_{39}H_{57}N_2O_{11}PNa$ [M + Na]$^+$ 783.3598, found 783.3617.

Dimethyl {*N*-[*p*-(*N*-methyl)aminobenzoyl]-α-methyl-L-glutamyl-γ-[Ψ{P(O)(OCH$_3$)CH$_2$ }]-glutarate (51). To a solution of the tetramethyl phosphinic pseudopeptide **50a** (60 mg, 0.09 mmol) in 5 mL of fresh-distilled THF was added 12 mg of 5% Pd/C, and the mixture was saturated with H_2. The resulting mixture was then stirred at room temperature under H_2 overnight. The Pd/C was filtered off. The filtrate was collected and concentrated to give 45 mg (100%) of **51** as an oil, which was used without further purification. ^{1}H NMR ($CDCl_3$): δ 7.75 (d, J = 12.2 Hz, 2H), 7.25−7.34 (m, 1H), 6.57 (d, J = 12.2 Hz, 2H), 4.79 (m, 1H), 4.36 (m, 1H), 3.77 (s, 3H), 3.61−3.72 (m, 9H), 2.86 (s, 3H), 2.31−2.33 (m, 2H), 2.27−2.29 (m, 2H), 2.23−2.25 (m, 1H), 1.93−1.98 (m, 3H), 1.82−1.87 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 174.91, 173.25, 172.85, 167.65, 152.61, 129.41, 121.59, 111.63, 52.93, 52.54, 52.13, 51.85, 39.00, 38.83, 31.50, 30.55, 29.73, 29.21, 29.05, 24.93. ^{31}P NMR ($CDCl_3$): δ 57.20, 56.71. ESI-HRMS (*m*/*z*): calcd for $C_{22}H_{33}N_2O_9PNa$ [M + Na]$^+$ 523.1821, found 523.1824.

Dimethyl {*N*-[(4-deoxy-2,4-diamino-10-methyl)pteroyl]-α-methyl-L-glutamyl-γ}-[Ψ{P(O)(OH)CH$_2$}]-glutarate (1d). Dibromotriphenylphosphine (127 mg, 0.3 mmol) was suspended in 1 mL of dry dimethylacetamide, followed by addition of (2,4-diamino-6-pteridinyl)methanol hydrochloride (**11a**) (23 mg, 0.1 mmol). The resulting mixture turned clear and red in few minutes with emission of gas. The reddish solution was stirred at room temperature for 20 h before diethylisopropylamine (52 mg, 0.4 mmol) and a solution of the tetramethyl ester **51** (45 mg, 0.09 mmol) in dimethylacetamide (1 mL) was added. The resulting mixture was then kept at 60 °C for 40 h and monitored by HPLC. The solvent was removed in a vacuum to give 240 mg of the crude product, which was purified by silica gel column chromatography (MeOH:CH_2Cl_2 = 1:1, R_f = 0.2) to afford 37 mg of a yellow solid. The yellow solid was then stirred for 3 h with 40 mg of Dowex-H in MeOH. Removal of the resin and solvent gave 25 mg (42% over three steps) of **1d** as a yellow solid. ^{1}H NMR (MeOH-d_4): δ 8.58 (s, 1H), 7.75 (d, J = 14.7 Hz, 2H), 6.78 (d, J = 14.7 Hz, 2H), 4.85 (s, 2H), 4.53−4.54 (m, 1H), 3.72 (s, 3H), 3.60−3.69 (m, 6H), 3.25 (s, 3H), 2.80 (m, 1H), 2.32−2.33 (m, 2H), 2.21 (m, 1H), 2.08 (m, 2H), 1.97−1.98 (m, 1H), 1.88−1.93 (m, 1H), 1.70 (m, 3H). ^{13}C NMR (MeOH-d_4): δ 174.95, 172.48, 171.81, 167.75, 162.16, 161.10, 152.37, 150.62, 147.70, 146.97, 127.87, 120.78, 119.70, 110.08, 54.03, 53.21, 52.68, 50.13, 49.83, 49.57, 41.18, 38.52, 37.17, 31.64, 29.80, 27.63, 27.55, 26.10, 25.38, 23.11. ^{31}P NMR (MeOH-d_4): δ 42.48. ESI-HRMS (*m*/*z*): calcd for $C_{28}H_{37}N_8O_9PNa$ [M + Na]$^+$ 683.2319, found 683.2328. UV λ_{max}: (phosphate buffer, pH 7.0) 225, 260, 310, 375 nm. Analytical HPLC method B: t_R = 23.6 min. Anal. ($C_{28}H_{37}N_8O_9P\cdot 3CH_3OH$) C, H, N.

***N*-{[4-(*N*-benzyloxycarbonyl-*N*-methyl)amino]benzoyl}-L-glutamyl-γ}-[Ψ{P(O)(OH)CH$_2$}]-glutaric Acid (52).** To a solution of tri-*tert*-butyl phosphinate **50b** (22 mg, 0.03 mmol) in 0.5 mL of freshly distilled CH_2Cl_2 was added TFA (0.1 mL, 1.2 mmol) at 0 °C. The resulting solution was warmed to room temperature and stirred overnight. Removal of the solvent in a vacuum gave the free tetraacid as an oily solid (yield 17 mg, 100%). ^{1}H NMR (MeOH-d_4): δ 7.90 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.31−7.37 (m, 5H), 7.04 (d, J = 8.1 Hz, 1H), 5.17 (s, 2H), 4.67 (dd, J_1 = 8.4 Hz, J_1 = 4.3 Hz, 1H), 3.35 (s, 3H), 2.82 (m, 1H), 2.38−2.43 (m, 2H), 2.28−2.36 (m, 2H), 2.13−2.14 (m, 1H), 1.90−2.10 (m, 5H). ^{13}C NMR (MeOH-d_4): δ 176.67, 175.46, 173.96, 173.80, 173.62, 168.89, 168.74, 155.86, 146.66, 136.72, 131.48, 129.56, 129.23, 128.58, 128.38, 128.22, 127.96, 125.46, 67.81, 53.82, 53.73, 53.61, 38.93, 36.89, 32.06, 31.18, 31.11, 30.30, 29.75, 28.93, 26.38, 25.66, 23.95. ^{31}P NMR (MeOH-d_4): δ 53.39. MS (*m*/*z*, M + Na$^+$): 601.

Methyl (2*S*)-2-*N*-[*p*-(*N*-carbobenzyloxy-*N*-methyl)aminobenzoyl]amino-4-phenylselenylbutyrate (54). To the suspension of diphenyl diselenide (463 mg, 1 mmol) in 15 mL of EtOH was added sodium borohydride (95 mg, 2.5 mmol) in portions under argon, until the orange color of the mixture disappeared. The solution of bromide **41** (1.19 g, 3.2 mmol) in 20 mL of EtOH was then added. After 30 min, the resulting mixture was poured into 200 mL of Et_2O. The resulting mixture was washed with $NaHCO_3$ and brine and dried over Na_2SO_4. Removal of the solvent gave 620 mg of the crude product, which was purified by flash chromatography (hexanes:EtOAc = 1:9, R_f = 0.45) to give 483 mg (90%) of **54** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.79 (d, J = 8.6 Hz, 2H), 7.49−7.50 (m, 2H), 7.31−7.38 (m, 7H), 7.24−7.27 (m, 3H), 7.07 (d, J = 7.8 Hz, 1H), 5.20 (s, 2H), 4.91−4.95 (m, 1H), 3.75 (s, 3H), 3.35 (s, 3H), 2.94−3.01 (m, 2H), 2.35−2.40 (m, 1H), 2.18−2.22 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 172.88, 166.90, 155.45, 146.70, 136.64, 133.22, 131.07, 130.04, 129.61, 128.97, 128.58, 128.35, 128.27, 127.59, 125.38, 68.09, 53.23, 53.03, 37.77, 33.40, 23.68. MS (*m*/*z*, M + Na$^+$): 563. Anal. ($C_{27}H_{28}N_2O_5$): C, H, N.

(2*S*)-2-*N*-[*p*-(*N*-carbobenzyloxy-*N*-methyl)aminobenzoyl]amino-4-phenylselenylbutyric Acid (55). To a solution of the methyl ester **54** (390 mg, 0.72 mmol) in 4 mL of MeOH and 1 mL of CH_2Cl_2 at 0 °C was added 1 mL of 1 N LiOH. The resulting mixture was stirred at room temperature for 2 h. Dowex-H was added to neutralize the reaction mixture and was then removed by filtration. The filtrate was collected and concentrated in vacuo to yield 380 mg (100%) of **55**, which was used without further purification. ^{1}H NMR ($CDCl_3$): δ 7.69 (d, J = 7.0 Hz, 2H), 7.29−7.34 (m, 7H), 7.10−7.16 (m, 5H), 5.15 (s, 2H), 4.64 (m, 1H), 3.22 (s, 3H), 2.89 (m, 2H), 2.27 (m, 1H), 2.13 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 168.50, 155.53, 146.47, 136.56, 132.54, 130.82, 129.48, 128.95, 128.58, 128.52, 128.35, 127.11, 125.14, 68.13, 53.40, 37.57, 32.74, 4.07. ESI-MS (*m*/*z*, [M−H$^+$ + Na$^+$]): 548.9.

Pivaloyloxymethyl (2*S*)-2-*N*-[*p*-(*N*-carbobenzyloxy-*N*-methyl)-aminobenzoyl]amino-4-phenylselenylbutyrate (56). To a solution of the acid **55** (273 mg, 0.52 mmol) in 5 mL of dry DMF was added POMI (630 mg, 2.6 mmol), dimethyl methylphosphonate (322 mg, 2.6 mmol), and Ag_2CO_3 (360 mg, 1.3 mmol). The

resulting mixture was allowed to stir at room temperature overnight and quenched with 2 mL of 2 N HCl. The precipitate was removed by filtration. The filtrate was collected and concentrated to give 483 mg of the crude product, which was purified by flash chromatography (hexanes:EtOAc = 2:1, R_f = 0.45) to yield 276 mg (83%) of **56** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.78 (d, J = 8.6 Hz, 2H), 7.50−7.52 (m, 2H), 7.32−7.39 (m, 7H), 7.26−7.29 (m, 3H), 6.78 (d, J = 7.7 Hz, 1H), 5.89 (d, J = 5.5 Hz, 1H), 5.78 (d, J = 5.5 Hz, 1H), 5.21 (s, 2H), 4.95−4.99 (m, 1H), 3.38 (s, 3H), 2.97 (m, 2H), 2.34−2.41 (m, 1H), 2.16−2.24 (m, 1H), 1.22 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.30, 171.19, 166.82, 155.44, 146.89, 136.61, 133.49, 130.89, 129.64, 128.96, 128.58, 128.37, 128.19, 127.76, 125.46, 80.43, 68.11, 53.12, 39.16, 37.76, 33.21, 27.24, 23.46. MS (*m*/*z*, M + Na^+): 663. Anal. ($C_{32}H_{36}N_2O_7Se$): C, H, N.

Pivaloyloxymethyl (2*S*)-*N*-[*p*-(*N*-carbobenzyloxy-*N*-methyl)-aminobenzoyl]-2-vinylglycine (57). To a solution of the POM ester **56** (276 mg, 0.43 mmol) in 25 mL of freshly distilled THF was added 0.5 mL of H_2O_2 (30% in H_2O). The resulting mixture was heated at reflux temperature for 1 h, diluted with 200 mL of EtOAc, washed with brine, and then dried over Na_2SO_4. Removal of the solvent gave 210 mg of the crude product, which was purified by flash chromatography (hexanes:EtOAc = 2:1, R_f = 0.4) to yield 187 mg (90%)of 57 as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.82 (d, J = 8.6 Hz, 2H), 7.29−7.37 (m, 7H), 6.92 (d, J = 7.4 Hz, 1H), 5.95−6.01 (m, 1H), 5.91 (d, J = 5.5 Hz, 1H), 5.80 (d, J = 5.5 Hz, 1H), 5.44 (dd, J_1=17.1 Hz, J_2=1.4 Hz, 1H), 5.36−5.38 (m, 1H), 5.34 (dd, J_1=10.4 Hz, J_2=1.4 Hz, 1H), 5.19 (s, 2H); 3.36 (s, 3H), 1.22 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 177.28, 169.89, 166.57, 155.45, 146.85, 136.60, 131.80, 130.84, 128.94, 128.56, 128.34, 128.27, 125.44, 119.14, 80.33, 68.08, 55.26, 39.18, 37.74, 27.23. ESI-HRMS (*m*/*z*): calcd for $C_{26}H_{30}N_2O_7Na$ [M + Na]$^+$ 505.1951, found 505.1950.

Bis(pivaloyloxymethyl) {*N*-{*p*-[(*N*-carbobenzyloxy-*N*-methyl)-amino]benzoyl}-α-pivaloyloxymethyl-L-glutamyl-γ}-[Ψ{P(O)(OCH$_3$)CH$_2$}]-glutarate (59). To a solution of the POM ester **57** (73 mg, 0.15 mmol) and ammonium hypophosphite (38 mg, 0.45 mmol) in 1 mL of MeOH was added triethylborane (1 M in hexanes, 0.18 mL, 0.18 mmol). The resulting mixture was stirred open to the air at room temperature for 3 h. The solvent was then removed on a rotary evaporator while the temperature of the water bath was kept lower than 30 °C. $KHSO_4$ (2 mL, 1 M) was added to the residue. The resulting aqueous mixture was extracted with EtOAc (10 mL × 3). The organic extracts were combined and dried over Na_2SO_4. Removal of the solvent gave 82 mg of the desired product **58**, which was used in the next step without further purification.

To the solution of the phosphinic **58** obtained above in 1 mL of freshly distilled CH_2Cl_2 was added the solution of bis(pivaloyloxymethyl) α-methyleneglutarate **34** (140 mg, 0.38 mmol) in 1 mL of freshly distilled CH_2Cl_2 dried over $MgSO_4$ through a syringe with a filter under argon, followed by addition of BSA (153 mg, 0.75 mmol). The resulting solution was heated at reflux temperature overnight and then quenched with 2 N HCl (2 mL). The mixture was stirred vigorously for 3 h and diluted with 50 mL of EtOAc (10 mL × 3). The resulting mixture was washed with brine and dried over Na_2SO_4. Removal of the solvent gave 61 mg of the crude product as a light yellow oil, which was used in the next step without further purification.

The product obtained above was dissolved in 5 mL of EtOAc, followed by addition of the solution of CH_2N_2 in Et_2O until the color of the mixture remained green yellow. The resulting mixture was then stirred at room temperature for another hour. A drop of AcOH was added to quench the reaction. Removal of the solvent gave 70 mg of the crude product, which was purified by flash chromatography (EtOAc:hexanes = 2:1, R_f = 0.4) to give 65 mg (46% over 3 steps) of **59** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.93−8.00 (m, 1H), 7.91 (d, J = 8.4 Hz, 2H), 7.29−7.36 (m, 7H), 5.61−5.89 (m, 6H), 5.17 (s, 2H), 4.75−4.79 (m, 1H), 3.62−3.73 (m, 3H), 3.34 (s, 3H), 2.86−2.87 (m, 1H), 2.35−2.40 (m, 2H), 2.22−2.27 (m, 2H), 2.18−2.21 (m, 1H), 1.97−2.03 (m, 3H), 1.82−1.88 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 177.42, 177.36, 177.33, 172.94, 172.89, 171.34, 171.30, 170.73, 170.67, 167.12, 167.05, 155.42, 146.72, 136.66, 130.73, 128.90, 128.48, 128.28, 125.34, 80.49, 80.31, 80.19, 79.98, 67.99, 53.38, 53.13, 53.05, 52.93, 52.03, 51.82, 39.23, 39.11, 38.79, 38.66, 37.74, 31.20, 31.15, 31.11, 30.48, 30.11, 29.73, 29.40, 28.59, 28.51, 28.07, 27.61, 27.22, 25.31, 24.87, 24.58, 24.28, 24.14, 23.95, 23.77. ^{31}P NMR ($CDCl_3$): δ 57.18, 57.04, 56.61. ESI-HRMS (*m*/*z*): calcd for $C_{45}H_{63}N_2O_{17}PNa$ [M + Na]$^+$ 957.3762, found. 957.3786.

Bis(pivaloyloxymethyl) {*N*-[*p*-(*N*-methyl)aminobenzoyl]-α-pivaloyloxymethyl-L-glutamyl-γ}-[Ψ{P(O)(OCH$_3$)CH$_2$}]-glutarate (60). To a solution of the tri-POM ester **59** (56 mg, 0.06 mmol) in 10 mL of freshly distilled THF was added 12 mg of Pd/C. The resulting mixture was then stirred overnight under H_2 at room temperature, after which the Pd/C was removed by filtration. The filtrate was collected and concentrated to give the 40 mg of the crude product, which was purified by the preparative TLC (EtOAc:hexanes = 4:1, R_f = 0.6) to yield 35 mg (73%) of **60** as a colorless oil. ^{1}H NMR ($CDCl_3$): 7.75 (d, J = 8.4 Hz, 2H), 7.37−7.42 (m, 1H), 6.58 (d, J = 8.4 Hz, 2H), 5.81−5.88 (m, 3H), 5.68−5.80 (m, 5H), 4.77−4.81 (m, 1H), 3.63−3.74 (m, 3H), 2.87 (s, 3H), 2.87 (m, 1H), 2.35−2.42 (m, 2H), 2.22−2.27 (m, 2H), 2.08−2.14 (m, 1H), 1.90−2.00 (m, 3H), 1.77−1.88 (m, 2H), 1.19−1.21 (m, 27H). ^{13}C NMR ($CDCl_3$): δ 177.48, 177.41, 177.37, 173.09, 173.04, 173.01, 172.96, 171.39, 171.34, 171.12, 171.10, 167.79, 167.76, 167.71, 152.66, 152.62, 129.50, 121.57, 121.54, 121.49, 111.73, 80.54, 80.48, 80.33, 80.30, 80.25, 79.99, 53.13, 52.98, 52.93, 52.84, 52.01, 51.96, 51.83, 51.79, 51.73, 39.12, 38.77, 38.71, 38.61, 31.23, 31.17, 31.14, 30.59, 30.43, 30.07, 29.71, 29.28, 28.60, 28.51, 27.61, 27.23, 24.57, 24.47, 24.28. ^{31}P NMR ($CDCl_3$): δ 57.00, 56.89, 56.47. ESI-HRMS (*m*/*z*): calcd for $C_{37}H_{57}N_2O_{15}PNa$ [M + Na]$^+$ 823.3394, found 823.3395.

Bis(pivaloyloxymethyl) {*N*-[(4-deoxy-2,4-diamino-10-methyl)-pteroyl]-α-pivaloyloxymethyl-L-glutamyl-γ}-[Ψ{P(O)(OCH$_3$)-CH$_2$}]-glutarate (1b). To the suspension of dibromotriphenylphosphine (63 mg, 0.15 mmol) in 0.5 mL of *N*,*N*-dimethylacetamide was added (2,4-diamino-6-pteridinyl)methanol hydrochloride (**11a**) (12 mg, 0.05 mmol). The resulting mixture turned to a red homogeneous solution in a few minutes with emission of gas. The reddish solution was stirred at room temperature for 18 h before diisopropylethylamine (26 mg, 0.2 mmol) was added. To the resulting mixture was added a solution of the POM ester **60** (40 mg, 0.05 mmol) in dimethylacetamide (1 mL). The resulting mixture was then kept at 60 °C for 2 days and reaction progress was monitored by analytical HPLC. After the reaction was completed, the solvent was removed in a vacuum. The residue was dissolved in 100 mL of EtOAc and washed with 1 N HCl (5 mL × 3) and brine (5 mL × 3) and then dried over Na_2SO_4. Removal of the solvent gave 116 mg of a brown oily solid as the crude product, which was dissolved in 5 mL of EtOAc and treated with CH_2N_2 (∼1 mmol). Concentration of the reaction mixture gave 120 mg of the crude product, which was purified by semipreparative HPLC to yield 20 mg (41%) of **1b** as a yellow oil. ^{1}H NMR (DMSO-d_6): δ 8.56 (s, 1H), 8.47, (d, J = 7.0 Hz), 7.72 (d, J = 8.5 Hz, 2H), 7.72 (bs, 1H), 7.48 (bs, 1H), 6.82 (d, J = 8.5 Hz, 2H), 6.67 (bs, 2H), 5.65−5.76 (m, 6H), 4.78 (s, 2H), 4.37−4.38 (m, 1H), 3.52 (d, J = 10.5 Hz, 3H), 3.21 (s, 3H), 2.75 (m, 1H), 2.34−2.40 (m, 2H), 2.04−2.10 (m, 1H), 1.85−1.91 (m, 4H), 1.78−1.85 (m, 3H), 1.17 (s, 9H), 1.12 (s, 9H), 1.10 (s, 9H). ^{31}P NMR (DMSO-d_6): δ 55.80, 55.76, 55.63, 55.56. ESI-HRMS (*m*/*z*): calcd for $C_{44}H_{63}N_8O_{15}$-PNa [M + Na]$^+$ 997.4048, found 997.4053. UV λ_{max}: (0.1 N NaOH) 255, 306, 370 nm; (0.1 N HCl) 245, 308. Analytical HPLC method B: t_R = 25.8 min.

Bis(pivaloyloxymethyl) {*N*-[(4-deoxy-2,4-diamino-10-methyl)-pteroyl]-α-pivaloyloxymethyl-L-glutamyl-γ}-[Ψ{P(O)(OH)CH$_2$}]-glutarate (1c). To a suspension of dibromotriphenylphosphine (76 mg, 0.18 mmol) in 0.5 mL of *N*,*N*-dimethylacetamide was added (2,4-diamino-6-pteridinyl)methanol hydrochloride (**11a**) (14 mg, 0.06 mmol). The resulting mixture turned to a red homogeneous solution in a few minutes with emission of gas. The reddish solution was stirred at room temperature for 18 h before diisopropylethylamine (30 mg, 0.23 mmol) was added. To the resulting mixture

was added a solution of the POM ester **60** (32 mg, 0.04 mmol) in dimethylacetamide (1 mL). The resulting mixture was then kept at 60 °C for 2 days and reaction progress was monitored by analytical HPLC. After the reaction was completed, the solvent was removed in a vacuum. The residue was dissolved in 100 mL of EtOAc and washed with 1 N HCl (5 mL × 3) and brine (5 mL × 3) and then dried over Na_2SO_4. Removal of the solvent gave 134 mg of a brown oily solid as the crude product, which was purified by preparative HPLC (method B) to yield 12 mg (31%) of **1c** as a yellow solid. ^{1}H NMR (DMSO-d_6): δ 9.28 (s, 1H), 9.08 (s, 1H), 8.72 (s, 1H), 8.64, (d, J = 6.5 Hz), 7.75 (d, J = 9.0 Hz, 2H), 7.56 (bs, 1H), 7.36 (bs, 1H), 6.82 (d, J = 9.0 Hz, 2H), 5.63−5.76 (m, 6H), 4.88 (s, 2H), 4.38 (m, 1H), 3.25 (s, 3H), 2.75 (m, 1H), 2.36 (m, 2H), 1.78−1.93 (m, 5H), 1.63−1.72 (m, 3H), 1.19 (s, 9H), 1.16 (s, 9H), 1.13 (s, 9H). ^{31}P NMR (DMSO-d_6): δ 47.11. ESI-HRMS (*m*/*z*): calcd for $C_{43}H_{61}N_8O_{15}PNa$ $[M + Na]^+$ 983.3892, found 983.3054. UV λ_{max}: (0.1 N NaOH) 255, 306, 370 nm; (0.1 N HCl) 245, 308. Analytical HPLC method B: t_R = 23.5 min.

Cytotoxicity and Hydrolysis Studies. Compounds **1a**−**d** and **2a**−**d** were dissolved in DMSO to provide 1 mM stock solutions. Serial dilutions were done either in DMSO (cell culture cytotoxicity) or phosphate (20 mM, pH 8.0) (hydrolysis studies). Cells were grown in RPMI 1640 cell culture medium (Sigma) as previously described[39] and exposed continuously to compounds for a period of 72 h. Cytotoxicity was determined by use of a Cell Titer-Blue Cell Viability kit (Promega), and IC_{50} data (Table 2) were obtained from dose−response curves obtained over a concentration range from 1 nM to 1 μM. For hydrolysis studies (Figures 3−5), the t = 0 points indicate the time a sample was applied to the HPLC column by the autosampler following dilution of the stock solution with phosphate buffer or cell line medium and filtration of the sample.

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Supporting Information Available: Experimental procedures and characterization of compound **46b**; combustion analysis results for compounds **1d**, **2b**, and **2d**; ^{1}H NMR spectral data for **1b**−**d**, **2b**, and **2d**; ^{13}C NMR spectral data for **1d**, **2b**, and **2d**; and ^{31}P NMR spectral data for **1b**−**d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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