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Design, Synthesis, and Biochemical Evaluation of Phosphonate and Phosphonamidate Analogs of Glutathionylspermidine as Inhibitors of Glutathionylspermidine Synthetase/Amidase from *Escherichia coli*

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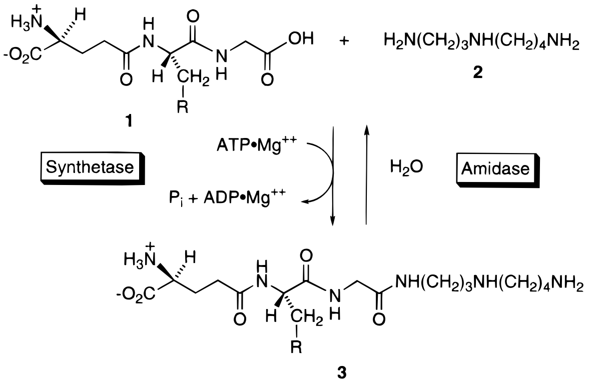
Three phosphapeptides designed to mimic two distinct tetrahedral intermediates formed during either the synthesis or hydrolysis of glutathionylspermidine (Gsp) were synthesized and evaluated as inhibitors of the bifunctional enzyme Gsp synthetase/amidase. While the polyamine-containing phosphapeptides were determined to be potent and selective inhibitors, they selectively inhibit the synthetase activity over the amidase domain. A phosphonate-containing tetrahedral mimic is a reversible mixed-type inhibitor of Gsp synthetase with an inhibition constant of 6 μM for the inhibitor binding to the free enzyme (K_i) and 14 μM for the inhibitor binding to the enzyme−substrate complex (K_i'). The corresponding phosphonamidate is a slow-binding inhibitor with a K_i of 24 μM and a K_i^* (isomerization inhibition constant) of 0.88 μM. A non-polyamine-containing phosphonamidate exhibits no significant inhibition of the synthetase or amidase activity.

Introduction

Parasitic diseases are the major killer of millions of children each year in the world. They are also the most common opportunistic infections that affect patients with acquired immunedeficiency syndrome (AIDS).[1,2] However, compared to bacterial diseases, the development of chemotherapy for the treatment of parasitic diseases[1,3] has been hindered by the close similarities between parasite and host metabolism. Most of the existing drugs are either ineffective or toxic to the host; some are carcinogenic.[1,2,4] Much effort has been made during the last decade to differentiate between parasite and host metabolism. It was first discovered that a major difference exists in the biochemistry of defense mechanisms against oxidative damage.[5] Unlike host cells which use a glutathione/glutathione reductase couple to maintain the intracellular thiol redox balance and therefore defend against oxidative stress, protozoal parasites of the genera *Trypanosoma* and *Leishmania* depend on a conjugate of glutathione and spermidine, namely, trypanothione (N^1,N^8-diglutathionylspermidine), for redox balance and oxidant defense.[5] Subsequent discovery of trypanothione reductase (TR),[6] a parasitic enzyme with considerable structural[7−10] and mechanistic[4,11] similarity to the host glutathione reductase, provides a means by which trypanothione can regulate the intracellular thiol redox balance in the parasite. The substrate specificity of TR and the unique presence of trypanothione in trypanosomatid parasites present two ideal targets for antiparasitic drug design.[4] Consequently, inhibition of TR has been actively investigated.[3,12] In addition, studies have demonstrated that trypanosomatid parasites lack the enzymes catalase and glutathione peroxidase and therefore may utilize trypanothione to detoxify H_2O_2 and other organic hydroperoxides through non-enzyme-catalyzed reactions.[3,13−15] Thus, interfering with the trypanothione biosynthetic pathway is also attractive for antiparasitic drug design.[16,17]

Glutathionylspermidine (Gsp) synthetase, isolated from *Crithidia fasciculata*[18,19] or *Escherichia coli*,[20] is a key enzyme that participates in the first of two similar steps of trypanothione biosynthesis (eq 1).[15] It is an ATP-dependent enzyme that couples the hydrolysis of ATP to the ligation of GSH (R = SH, **1a**) and spermidine (**2**) to form Gsp (R = SH, **3a**) and ADP.[20] The catalytic mechanism has been investigated.[19] Substrate specificity studies revealed that both glutamate and glycine residues of GSH are important for the recognition of substrate by Gsp synthetase, while the thiol group of the cysteine is not essential for recognition; e.g., the alanine-containing analog (R = H, **1b**) and ophthalmic acid (R = CH$_3$, **1c**) are also good substrates for Gsp synthetase.[18,21,22] Spermidine-binding site studies showed that Gsp synthetase from *E. coli* recognizes a (ω-aminoalkyl)-1,3-diaminopropane with a deprotonated N-1 amino group as the likely reacting species.[20] Surprisingly, the *E. coli* Gsp synthetase was discovered to be a bifunctional enzyme containing a hydrolytic activity.[20] This activity, either as part of the full-length



(1)

a, R = SH; **b**, R = H; **c**, R = CH$_3$

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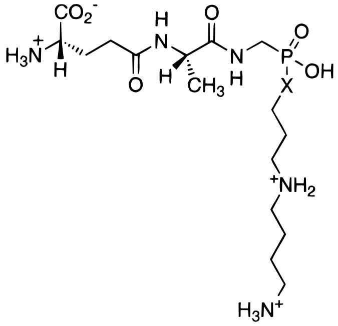
bifunctional protein or as a proteolytic fragment, is contained in a domain referred to as Gsp amidase.[21] Gsp amidase catalyzes the hydrolysis of Gsp to GSH and spermidine (eq 1). Substrate specificity studies for the Gsp amidase domain showed that this enzyme recognizes predominantly the glutathione portion of glutathionylspermidine.[21]

Although direct evidence is currently lacking, we postulate that the reaction catalyzed by Gsp synthetase proceeds through a tetrahedral intermediate, formed via an acyl phosphate, based on extensive literature precedent with other ATP-dependent peptide biosynthesis reactions.[23-25] Phosphonate, phosphonamidate, and phosphinates have been widely used as transition state analog inhibitors[26] of protease[27-30] or ATP-dependent ligase[31-33] enzymes by mimicking the proposed unstable tetrahedral intermediate in each case. We hypothesized that phosphonate (**4**) and phosphonamidate (**5**) peptides would be potent and specific inhibitors of Gsp synthetase/amidase and would interfere with the trypanothione biosynthetic pathway.[16] In addition, phosphapeptides such as **4** and **5** could act as inhibitors of the amidase activity by mimicking the tetrahedral intermediate formed as a result of direct attack of H_2O at the scissile amide bond.

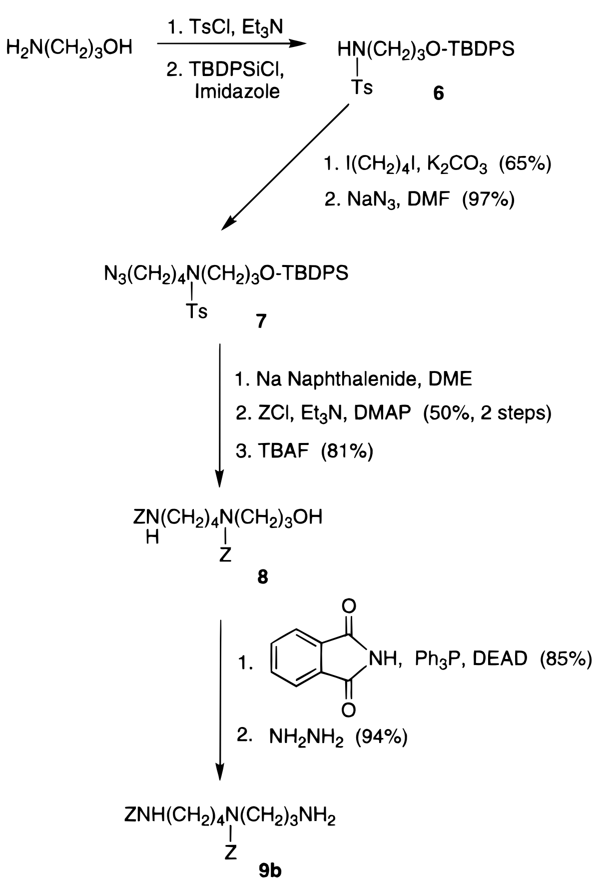


4, X = O
5, X = NH

Chemistry

In a previous paper, we described efforts made toward the synthesis of intermediates for the target molecules **4** and **5**.[16] Initially, the azido group was chosen as a precursor of the N-8 primary amino group, whereas the tosyl group was chosen to protect the N-4 secondary amino group in spermidine derivatives. Thus, suitably protected precursors of 1-hydroxy-1-desaminospermidine and spermidine were coupled in satisfactory yield with methyl hydrogen (phthalimidomethyl)phosphonate (activation with either oxalyl chloride or via the Mitsunobu method) to provide, respectively, blocked phosphonate and phosphonamidate precursors of **4** and **5**.[16] However, it soon became evident that the deprotection of such polyfunctionalized molecules was very difficult, especially in the presence of the obstinate *N*-tosyl group. As a result, all attempts to isolate the target molecule **4** using such a protective strategy were unsuccessful.[34] One possible reason for these failures is the harsh conditions required for removal of the tosyl group that may affect the P—O bond (phosphonate, **4**) and/or the P—N bond (phosphonamidate, **5**) in the target molecules. In order to solve this problem, we decided to choose protective groups that can be removed simultaneously at the final stage under neutral conditions, e.g., catalytic hydrogenolysis. Thus, in the approach reported herein, all the amines in the precursors were protected by carbobenzyloxy (Cbz, Z) groups while the carboxylic acids and phosphonic acid were masked as their benzyl esters.

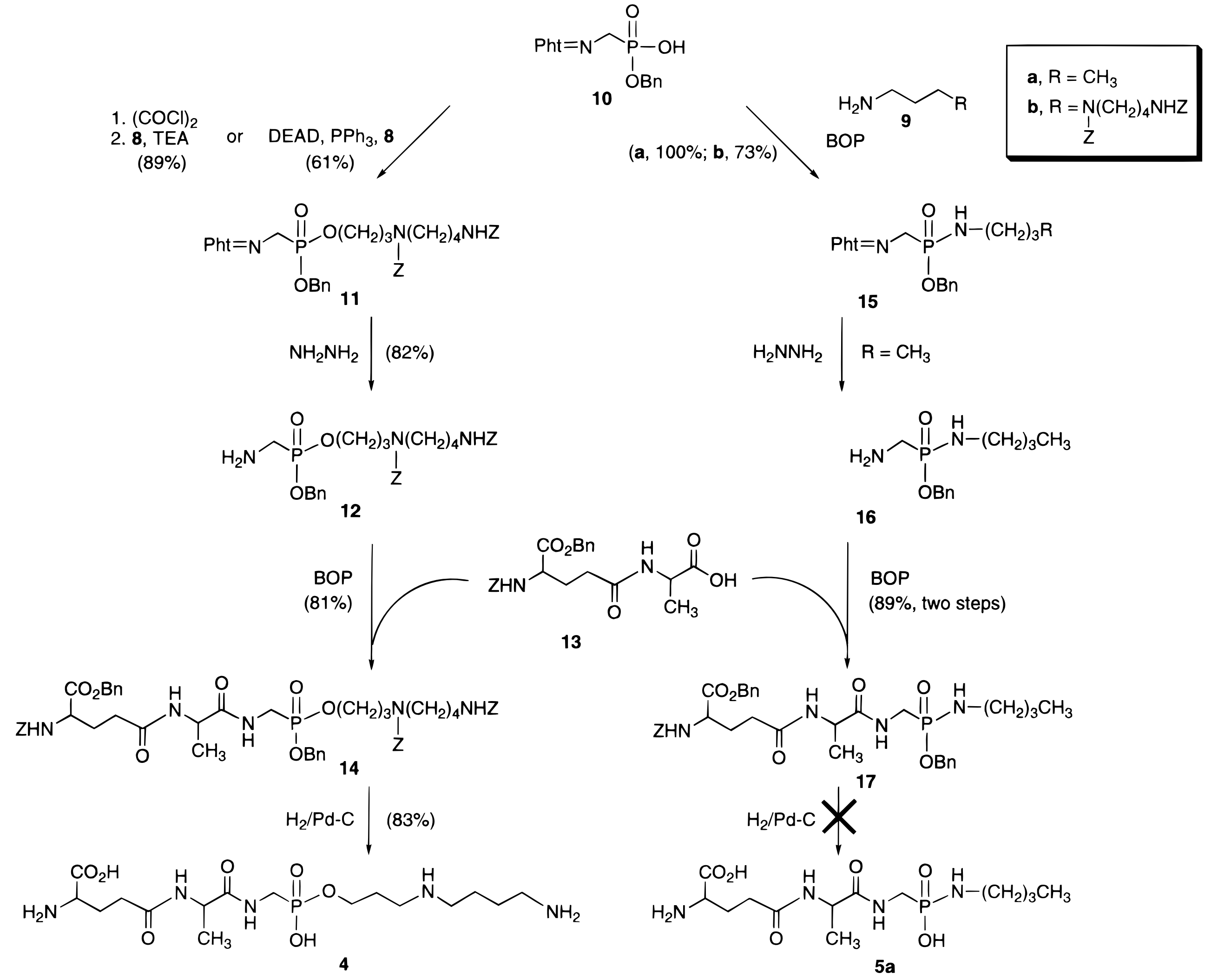
Scheme 1



The synthesis of the Cbz-containing, selectively protected derivatives of 1-hydroxy-1-desaminospermidine (**8**) and spermidine (**9b**) is outlined in Scheme 1. Starting with 3-amino-1-propanol, the synthesis of **8** was effected, via the orthogonally protected spermidine precursor 1-*O*-TBDPS-8-azido-4-azaoctane, **7**,[35] in seven steps with an overall yield of 20%. Selectively protected spermidine (**9b**) was obtained from **8** in two steps in 80% yield. Benzyl hydrogen (phthalimidomethyl)phosphonate (**10**) was prepared from (phthalimidomethyl)-phosphonic acid[36] and benzyl alcohol according to a general literature procedure.[37] As depicted in Scheme 2, coupling of **10** with **8** proceeded smoothly by either the oxalyl chloride approach[16] or the Mitsunobu method[16,38,39] to afford the mixed phosphonate **11** in 89% and 61% yields, respectively. Removal of the phthaloyl group in **11** by hydrazinolysis afforded **12** in 82% yield. Crude **12** could be used directly in the coupling with **13** to yield **14**. Initially, partial success was achieved by employing either DCC/HOBt or the mixed anhydride method by which **14** was obtained in only 43% and 24% yields, respectively. The BOP reagent, introduced initially for peptide synthesis,[40] proved to be much more efficient and reliable; a much higher yield (81%) of **14** was obtained by using this reagent in the coupling reaction.[41,42] Catalytic hydrogenolysis of **14** followed by cation exchange chromatography on AG 50W-X2 resin afforded the target phosphonate **4** in 83% yield.

In contrast to the facile coupling of **10** with **8** to form

Scheme 2



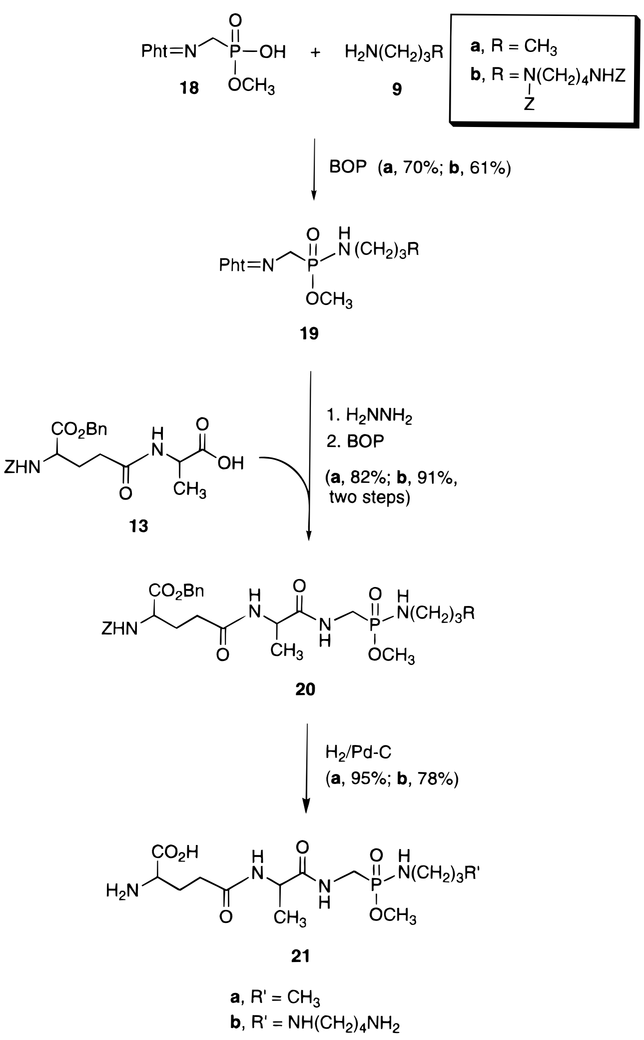
the complex phosphonate **11**, the coupling of **10** with **9** to form the corresponding phosphonamidate **15** proved to be very idiosyncratic. Although the phosphonic mono*methyl* ester corresponding to benzyl ester **10** coupled smoothly with simple amines, including a protected form of spermidine similar to **9b**, following generation of the corresponding phosphonochloridate,[16] the coupling of **10** with *N*-butylamine (**9a**) or **9b** using the oxalyl chloride method was not satisfactory. However, other amines such as 3-chloropropylamine and diethyl glutamate were coupled with **10** to provide the corresponding phosphonamidates in moderate to good yield under the same conditions (S. Chen, J. K. Coward, unpublished results). Fortunately, using the BOP method, **10** coupled smoothly with both **9a**,**b** to give **15a**,**b**, respectively, in satisfactory yields.

The lability of P−N bonds in phos*phon*amidates compared to those in phos*phor*amidates is well documented. The P−N bond in a phosphonamidate is hydrolyzed faster than that in a phosphoramidate.[43] Nonetheless, *N*-terminal protected phosphonamidate peptides, stabilized as the corresponding lithium salt, were successfully prepared as inhibitors of some protease enzymes.[28,44] However, in the case of completely deprotected phosphonamidate peptides, even the lithium salts could not be isolated due to their extreme lability.[45] In order to determine whether this is also the case for nonpeptidic P−N bonds, **17** was prepared from **15a** in a similar way as described for the synthesis of **14** (Scheme 2). Subsequent hydrogenolysis of this material in EtOH under mild basic conditions failed to give any trace of the desired phosphonamidate **5a**. Instead, a product resulting from ethanolysis of the P−N bond was isolated and characterized by NMR and MS analyses. Instability of the P−N bond is of concern for enzyme inhibitor and drug design. For instance, some phosphoramidates are known to be potent inhibitors of several proteases such as collagenase.[46] However, these phosphoramidates could not be developed as useful drugs.[47] Considering the instability of **5** and literature precedent for the use of methyl or phenyl phosphonamidates as inhibitors of serine proteases such as chymotrypsin,[48] we attempted to stabilize the target molecule **5** by blocking the free hydroxyl group on phosphorus as a methyl ester. Therefore, esters **21a,b** were synthesized (Scheme 3) as surrogates of **5** for inhibition studies. Intermediates **19a**,**b** were obtained in reasonable yields from **18**[16] and **9a**,**b**, respectively, using either the BOP or oxalyl chloride method. The desired methyl phosphonamidates, **21a**,**b**, were obtained from **19a**,**b** by chemistry similar to that described for the synthesis of **4**.

Biochemical Evaluation

Compounds **4** and **21a**,**b** were assayed for inhibition of the *E. coli* Gsp synthetase using a pyruvate kinase/

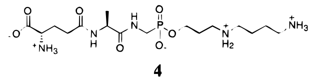
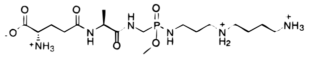
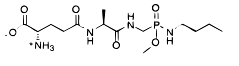
Scheme 3



lactate dehydrogenase coupled assay, as previously described.[20,21] As noted above, the *E. coli* enzyme is a bifunctional protein; the second activity, Gsp amidase, catalyzes the hydrolysis of Gsp back to glutathione and spermidine. Amidase and synthetase activities are contained in separate *N*-terminal and *C*-terminal domains, respectively, of the 70 kDa protein.[21] Therefore, the inhibition of Gsp amidase was also investigated using an alcohol dehydrogenase/aldehyde dehydrogenase coupled assay, as previously described.[21] To avoid the potential artifacts due to negative regulation of the amidase activity by the synthetase domain, the 225-amino acid amidase fragment (25 kDa)[21] was used in all amidase inhibition studies.

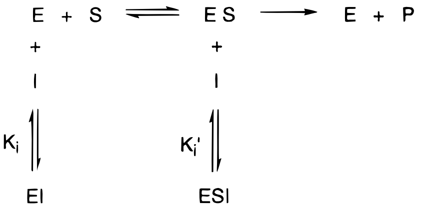
Inhibition results of Gsp synthetase are summarized in Table 1, and several initial observations are noteworthy. First, the spermidine portion is essential for inhibition. Compound **21b**, containing the spermidine moiety, is much more potent than **21a**, which has an *N*-butyl group substituted for spermidine. Presumably, these results reflect a binding preference which has been also observed for spermidine analogs[20] and Gsp-phosphinate analogs.[49] Second, the phosphonamidate **21b**, the more stable surrogate of **5**, was observed by HPLC to decompose gradually during inhibition assays (30 min, 37 °C). The observed instability is consistent with that observed for phosphonamidate inhibitors of D-Ala-D-Ala ligase.[50,51]

Table 1. Inhibition of Gsp Synthetase by **4** and **21a,b**

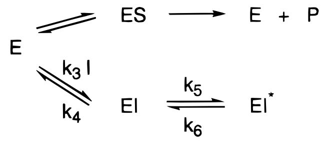
Compounds	Inhibition of Gsp Synthetase[a]	Inhibition of Gsp Amidase[b]
 **4**	$K_i = 6.0\ \mu M$, $K_i' = 14\ \mu M$[c]	not detectable
 **21b**	$K_i = 24\ \mu M$, $K_i^* = 0.88\ \mu M$	3.4%
 **21a**	5.7% ([I] = 1.0 mM), 31.3% ([I] = 7.3 mM)	1.8%

[a] For definition of inhibition constants, see Schemes 4 and 5. [b] Percent inhibition at 1 mM inhibitor. [c] See ref 21.

Scheme 4



Scheme 5



$$K_i = k_4 / k_3$$

$$K_i^* = K_i\ k_6 / (k_5 + k_6)$$

The phosphonate and phosphonamidates all exhibit ATP-independent inhibition; i.e., phosphorylation of these inhibitors does not occur, in contrast to ATP-dependent inhibition by a closely related phosphinate inhibitor, which has been interpreted as reflecting formation of an enzyme-bound phosphinophosphate.[49] The phosphonate **4** was determined to exhibit noncompetitive or mixed-type inhibition (Scheme 4),[21] whereas the phosphonamidate **21b** was shown to be a time-dependent (slow-binding) inhibitor. The observed 30-fold tightening ($K_i \rightarrow K_i^*$) is typically interpreted as an isomerization of a collisional E·I complex to a rearranged E·I* complex (Scheme 5).[52] It is possible, however, that some portion of the slow-binding inhibition is due to a slow decomposition of **21b** (*vide supra*) and more potent inhibition by the product(s).

For inhibition of the Gsp amidase domain, we hypothesized[21] that the synthesized phosphapeptides would mimic the tetrahedral intermediate that would result if the hydrolysis involved direct attack of H_2O (e.g., zinc or aspartyl proteases). Poor inhibition, orders of magnitude less than observed for the synthetase domain (Table 1), argues against such a reaction mechanism and suggests an amidase mechanism of covalent catalysis by a serine or cysteine nucleophile forming an acyl—enzyme intermediate.[21] Recent studies using rapid kinetic methods and mutagenesis indicate that such a covalent acyl-enzyme mechanism does apply; cysteine appears to be a likely candidate for the nucleophile (C. T. Walsh, et al. *Biochemistry*, in press).

In summary, the phosphapeptides **4** and **21b** selectively inhibit Gsp synthetase with inhibition constants in the micromolar range. Taken together with data on the corresponding phosphinate,[49] where X in **4** is CH_2 rather than O, these studies define the structural determinants for effective design of potent inhibitors of Gsp synthetase. Unfortunately, neither **4** nor **21b** is an effective inhibitor of the growth of *Tryanoma brucei* (C. Bacchi, personal communication) or *Leishmania donovani* and *Trypanoma cruzi* (S. Croft, personal communication) in cell culture assays. A variety of structural modifications can be envisioned that may allow for these phosphapeptides to penetrate the cell membranes. Synthetic studies to access such modified tetrahedral mimics are currently underway in our laboratory.

Experimental Section

General Methods. ^{1}H NMR spectra were recorded at 300 or 360 MHz on Bruker spectrometers. Chemical shifts in ppm were measured relative to TMS. All coupling constants are reported in hertz (Hz). ^{13}C NMR spectra were recorded on a Bruker 360 spectrometer at 90.6 MHz or a Bruker 300 spectrometer at 75.5 MHz, and chemical shift data are reported in reference to TMS. When appropriate, carbon−phosphorus coupling is reported with the chemical shift data (multiplicity, coupling constant in Hz). ^{31}P NMR spectra were recorded on a Bruker 360 spectrometer at 145 MHz with 85% H_3PO_4 ($\delta = 0$ ppm) as an external reference and with broad-band ^{1}H decoupling. All NMR spectra were obtained at room temperature (ca 298 K) unless otherwise indicated. Mass spectra and high-resolution mass spectra were recorded on a Finnigan 4500 GC/MS-EICI system or on a VG Analytical system, Model 70-250S. Elemental analyses were obtained from Atlantic Microlab Inc., at Norcross, GA, or at the Elemental Analysis Labs, Department of Chemistry, The University of Michigan. Melting points were determined using a Thomas-Hoover capillary apparatus and are uncorrected. Acetonitrile, DMF, ethylene glycol dimethyl ether (DME), and benzyl alcohol were distilled and stored over molecular sieves (4 Å). *N*-Methylmorpholine (NMM) and triethylamine (TEA) were stored over KOH pellets after distillation. Tetrahydrofuran (THF) was distilled under N_2 from violet sodium benzophenone ketyl before use. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride. Ethyl acetate (EtOAc), hexane, and other solvents were Baker HPLC grade and used without further treatment except as otherwise indicated. Commercially available amino acid and dipeptide precursors were purchased from Bachem-Bioscience Inc. or Fisher Scientific. Other reagents were obtained from Aldrich or as otherwise indicated. Compound **6** was prepared according to the procedures of Lakanen.[35] Synthesis of **7** was carried out by a slight modification of the original procedure[35] as detailed in the Supporting Information. The synthesis of Z-Glu-γ-Ala-OH (**13**) was carried out by standard methods and is described in the Supporting Information. Methyl hydrogen (phthalimidomethyl)phosphonate (**18**) was synthesized as previously described.[16]

***N*-(Benzyloxycarbonyl)-*N*′-(benzyloxycarbonyl)-*N*′-(3-hydroxypropyl)-1,4-diaminobutane (8).** To a stirred solution of **7** (3.0 g, 5.3 mmol) in dry DME (30 mL) in an oven-dried round bottom flask under dry nitrogen was added 1 M sodium naphthalenide-DME[53] solution dropwise at −78 °C (dry ice−2-propanol) until a green color persisted for at least 5 min. The resultant green solution was stirred at −78 °C for 1 h, the reaction was then quenched with water (2 mL), and solvent was removed by concentration under reduced pressure; the residue was partitioned between ethyl ether (50 mL) and water (50 mL). The organic layer was separated and washed with water (30 mL), 5% $NaHCO_3$ solution (40 mL), and brine (40 mL), dried over sodium sulfate, and then evaporated to dryness. The crude secondary amine, obtained as a semisolid material with naphthalene contamination, was thoroughly dried over P_2O_5 in vacuo and then dissolved, with triethylamine (3.7 mL, 26.5 mmol) and DMAP (0.3 g), in dry CH_2Cl_2 (80 mL) at 0 °C. To this solution was added dropwise a solution of CbzCl (3.01 mL, 21.2 mmol) in CH_2Cl_2 (10 mL) during a period of 30 min. The resultant yellow solution was stirred at room temperature for 24 h before being diluted with 150 mL of CH_2Cl_2, then washed with water (100 mL), 5% citric acid (100 mL), 5% $NaHCO_3$ solution (100 mL), and brine (100 mL), and dried over Na_2SO_4. After removal of the organic solvent under reduced pressure, the crude product was purified by column chromatography on silica gel using hexane/EtOAc (7:3) as eluant: yield 1.7 g (50%) of an oil; ^{1}H NMR (DMSO-d_6, 334 K) δ 0.99 (s, 9 H), 1.28−1.37 (m, 2 H), 1.39−1.51 (m, 2 H), 1.65−1.74 (m, 2 H), 2.95−3.01 (m, 2 H), 3.17 (t, 2 H, J = 7.6), 3.30 (t, 2 H, J = 8.0), 3.64 (t, 2 H, J = 8.0), 5.00 (s, 2 H), 5.03 (s, 2 H), 6.89−7.01 (br, 1 H), 7.25−7.36 (m, 10 H), 7.37−7.44 (m, 6 H), 7.52−7.61 (m, 4 H); ^{13}C NMR ($CDCl_3$) δ 156.5, 156.1, 137.1, 135.6, 133.9, 129.8, 129.7, 128.6, 128.2, 128.0, 127.9, 127.8, 127.3, 67.1, 66.8, 61.7, 46.9, 44.1, 40.8, 32.4, 27.4, 27.1, 25.5, 19.5; MS (CI with methane/NH_3) *m/z* (rel intensity) 653 (MH^+, 100), 545 (45), 91 (58); HRMS (CI) calcd for $C_{39}H_{48}N_2O_5SiH$ (MH^+) 653.3411, found 653.3443.

A solution of this fully protected amino alcohol (0.4 g, 0.6 mmol) and TBAF (1 M solution in THF, 2.41 mL, 2.41 mmol) in THF (20 mL) containing acetic acid (1 mL) was stirred at room temperature for 6 h. The reaction mixture was then concentrated, and the residue was partitioned between CH_2Cl_2 (30 mL) and water (20 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 20 mL). Organic extracts were combined and dried over Na_2SO_4. After being concentrated and purified by column chromatography on silica gel using hexane/EtOAc (1:1) as eluant, 0.20 g (81%) of the desired product **8** was obtained as a colorless oil: R_f 0.24 (hexane/EtOAc (1:1)); ^{1}H NMR (DMSO-d_6, 332 K) δ 1.34−1.42 (m, 2 H), 1.42−1.53 (m, 2 H), 1.57−1.66 (m, 2 H), 2.95−3.03 (m, 2 H), 3.15−3.29 (m, 4 H), 3.34−3.43 (m, 2 H), 4,18−4.28 (br, 1 H), 5.01 (s, 2 H), 5.06 (s, 2 H), 6.95−7.08 (br, 1 H), 7.15−7.44 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 157.7, 156.6, 136.8, 129.9, 128.7, 128.3, 128.1, 127.3, 67.5, 66.9, 58.7, 46.8, 43.7, 40.8, 30.9, 27.4, 25.8, 21.6; MS (CI with CH_4/NH_3) *m/z* (rel intensity) 415 (MH^+, 87), 307 (100), 173 (37), 108 (26); HRMS (CI) calcd for $C_{23}H_{30}N_2O_5H$ (MH^+) 415.2233, found 415.2256.

***N*-(Benzyloxycarbonyl)-*N*′-(benzyloxycarbonyl)-*N*′-(3-aminopropyl)-1,4-diaminobutane (9b).** To a stirred solution of **8** (1.3g, 3.12 mmol), phthalimide (0.46g, 3.12 mmol), and Ph_3P (0.86g, 3.28 mmol) in dry THF (16 mL) under N_2 was added DEAD (0.53g, 3.12 mmol) gradually at room temperature. The resultant yellow solution was stirred at room temperature overnight and then concentrated under reduced pressure. After usual workup, the desired product was purified by column chromatography (98−33% hexanes in EtOAc) to give 1.45 g (85%) of the phthaloyl derivative as a clear oil: R_f 0.35 (1:1 Hex/EtOAc); ^{1}H NMR (DMSO-d_6, 331 K) δ 1.31−1.44 (m, 2 H), 1.45−1.53 (m, 2 H), 1.71−1.89 (m, 2 H), 2.95−3.04 (m, 2 H), 3.19−3.27 (m, 4 H), 3.57 (t, 2 H, J = 7), 5.01 (s, 2 H), 5.02 (s, 2 H), 6.98−7.07 (br, 1 H), 7.25−7.36 (m, 10 H), 7.78−7.85 (m, 4 H); ^{13}C NMR δ 168.4, 156.6, 156.2, 136.8, 134.1, 132.1, 128.6, 128.1, 128.0, 127.9, 123.3, 67.1, 66.6, 47.4 (46.8), 45.4 (45.0), 40.7, 36.6, 35.8, 31.5, 28.0 (27.6), 27.2, 26.0 (25.6).

A mixture of the phthaloyl derivative (1.55 g, 2.8 mmol) and $NH_2NH_2{\cdot}H_2O$ (1.4g, 28 mmol) in MeOH was stirred at room temperature for 48 h. The resultant precipitate was removed by filtration, and the filtrate was concentrated under vacuum. The residue was then partitioned between CH_2Cl_2 and NH_4OH. The two layers were separated, the aqueous layers were extracted by CH_2Cl_2, and the combined organic extracts were dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure afforded 1.1 g (94%) of the desired product **9b** as an oil: ^{1}H NMR (DMSO-d_6, 331 K) δ 1.43−1.54 (m, 2 H), 1.55−1.70 (m, 4 H), 2.63 (t, 2 H, J = 6.6), 3.07−3.15 (m, 2 H), 3.28−3.39 (m, 4 H), 5.13 (s, 2 H), 5.18 (s, 2 H), 7.08−7.19 (br, 1 H), 7.32−7.49 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 156.6, 156.4, 136.9, 136.8, 128.6, 128.2, 128.1, 128.0, 67.1, 66.7, 46.8 (46.4), 44.7, 40.8, 39.3, 33.4 (33.0), 27.3, 26.0 (25.6); MS (EI, 70 eV) *m/z* (rel intensity) 413 (M^+, 0.7), 149 (14), 91 (100); HRMS (EI, 70 eV) calcd for $C_{23}H_{31}N_3O_4$ 413.2315, found 413.2294.

This material was used without further purification in the synthesis of **15b** and **19b**.

Benzyl Hydrogen (Phthalimidomethyl)phosphonate (10). To a stirred solution of diethyl (phthalimidomethyl)phosphonate (4.0 g, 13.4 mmol) in dry CH_2Cl_2 (40 mL) was added TMSBr (8.0 g, 52 mmol) at room temperature, under N_2. After 12 h stirring at room temperature the reaction mixture was concentrated under reduced pressure. The residue was then triturated with MeOH (45 mL) and AcOH (1.25 mL). Precipitated product was collected and washed thoroughly with MeOH to give 2.82 g (100%) of (phthalimidomethyl)phosphonic acid: mp 302−304 °C dec (lit.[36] mp 274−277 °C). This material was used directly in the next step.

A stirred suspension of (phthalimidomethyl)phosphonic acid (1.0 g, 0.47 mmol) in dry DMF (10 mL) was chilled in a dry ice−acetone bath (ca. −35 °C). Thionyl chloride (0.51 mL, 0.7 mmol) was then added through a syringe, and the reaction mixture solidified over a period of 30 min. The bath temperature was allowed to rise to −25 °C when benzyl alcohol (2.5 mL) was added slowly through a dropping funnel as the solid mass dissolved. The resultant clear solution was stirred at 0 °C for 1 h and then at room temperature for 12 h. The volatile components were removed on a rotary evaporator, and the residue was dissolved in 5% aqueous $NaHCO_3$ (15 mL) which was extracted with EtOAc (2 × 10 mL). The aqueous solution was acidified to pH 2−3 with concentrated hydrochloric acid at 0 °C and extracted with EtOAc (4 × 20 mL). The EtOAc extracts were washed with brine (30 mL) and dried over sodium sulfate. The solid product obtained after removal of the solvent was crystallized from hexane/$CHCl_3$ to give 1.37 g (88%) of a white powder: mp 176.5−178 °C; R_f 0.25 ($CHCl_3$/MeOH (2:1)); ^1H NMR (DMSO-d_6) δ 4.0 (d, 2 H, $J = 10.9$), 5.01 (d, 2 H, $J = 7.2$), 7.20−7.40 (m, 5 H), 7.75−7.95 (m, 4 H); ^{13}C NMR (DMSO-d_6) δ 166.2, 136.0, 134.4, 131.3, 128.1, 127.7, 127.3, 123.0, 66.2 (d, $J = 5.51$), 34.5 (d, $J = 150.1$); ^{31}P NMR (D_2O/NaOD) δ 14.3. Anal. ($C_{16}H_{14}NO_5P{\cdot}0.5H_2O$) C, H, N.

***O*-Benzyl-*O*-[3-[*N*-[4-[*N*-(benzyloxycarbonyl)amino]butyl]-*N*-(benzyloxycarbonyl)amino]propyl](phthalimidomethyl)phosphonate (11). A. Oxalyl Chloride Method.** To a suspension of the phosphonic monoester 10 (0.14 g, 0.42 mmol) in dry CH_2Cl_2 (15 mL) was added oxalyl chloride (0.2 g, 1.65 mmol) slowly at room temperature. After 2 h stirring, the resultant clear solution was concentrated under reduced pressure to afford a white solid which was redissolved in dry toluene, reconcentrated, and dried in vacuo. The resulting crude phosphonochloridate (^{31}P NMR ($CDCl_3$) $\boldsymbol{\delta}$ 32) was used in the next step without further purification; it was dissolved in dry CH_2Cl_2 (10 mL) followed by the addition of **7** (0.12 g, mmol) and DMAP (10 mg) at 0 °C under N_2. The resultant solution was allowed to stir at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 (30 mL) and washed subsequently with H_2O (1 × 30 mL), 5% citric acid (2 × 30 mL), H_2O (30 mL), 5% $NaHCO_3$ solution (2 × 30 mL), H_2O (30 mL), and brine (30 mL). After being dried over sodium sulfate, the solvent was removed to afford 0.22 g of a colorless oil which was purified by column chromatography eluting with EtOAc; 0.19 g (89%) of **11** was obtained as an oil. Spectral properties are identical with those described for **11** prepared via method B.

B. Mitsunobu Method. To a stirred solution of **10** (0.11 g, 0.34 mmol) were added **8** (0.14 g, 0.34 mmol) and triphenylphosphine (88 mg, 0.34 mmol) in dry THF (10 mL) followed by dropwise addition of DEAD (63.3 mg, 0.37 mmol) in dry THF (2 mL) at room temperature. The resultant yellow solution was stirred at room temperature for 8 h and then concentrated under reduced pressure. The residue was dissolved in EtOAc (40 mL) and washed successively with 5% citric acid (2 × 20 mL), H_2O (20 mL), and brine (30 mL). Removal of the solvent after being dried over Na_2SO_4 afforded crude **11** as a dense oil which was purified by column chromatography eluting with 100% EtOAc; 0.15 g (61%) of **11** was obtained as an oil: ^1H NMR ($CDCl_3$) δ 1.32−1.60 (br, 4 H), 1.77−1.89 (br, 2 H), 3.11−3.28 (m, 6 H), 3.94−4.22 (m, 4 H), 5.04−5.28 (m, 7 H), 7.30 (s, 15 H), 7.64−7.73 (m, 2 H), 7.75−7.86 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 166.8, 156.5, 156.1, 136.9, 134.2, 131.9, 129.7, 128.6, 128.5, 128.2, 128.0, 127.9, 123.6, 68.4 (d, $J = 6$), 67.1, 66.6, 64.3, 47.5, 43.8, 40.8, 34.8, 32.7, 29.8, 27.2, 25.6; ^{31}P NMR ($CDCl_3$) δ 20.7; MS (CI with NH_3) *m/z* (rel intensity) 728 (MH^+, 6), 620 (11), 512 (23), 422 (58), 307 (100), 91 (37); HRMS (CI) calcd for $C_{39}H_{43}N_3O_9P$ (MH^+) 728.2737, found 728.2803. Anal. ($C_{39}H_{42}N_3O_9P{\cdot}0.5H_2O$) C, H, N.

***O*-Benzyl-*O*-[3-[*N*-[4-[*N*-(benzyloxycarbonyl)amino]butyl]-*N*-(benzyloxycarbonyl)amino]propyl](aminomethyl)phosphonate (12).** A solution of **11** (0.12 g, 0.16 mmol) and hydrazine monohydrate (0.12 mL, 1.6 mmol) in MeOH (8 mL) was stirred at room temperature for 24 h. The precipitated byproducts were removed by filtration, and the filtrate was concentrated to afford a solid material which was then partitioned between CH_2Cl_2 and water (20 mL each); the organic layer was separated while the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried over sodium sulfate and evaporated to dryness. The desired product, **12** (81 mg, 85%), was obtained as a colorless oil: R_f 0.2 (EtOAc); ^1H NMR ($CDCl_3$) δ 1.35−1.60 (m, 6 H), 1.80−1.91 (br, 2 H), 2.85−2.96 (m, 2 H), 3.15−3.35 (br, 6 H), 3.92−4.13 (m, 2 H), 5.05−5.11 (m, 6 H), 7.15−7.34 (m, 16 H); ^{13}C NMR ($CDCl_3$) δ 156.6, 156.2, 136.8, 136.4, 129.9, 128.8, 128.6, 128.4, 128.2, 128.0, 127.3, 67.9, 67.2, 66.7, 63.6, 53.6, 47.5, 44.4, 40.7, 38.6 (d, 148.5), 30.6, 27.2, 25.4; ^{31}P NMR ($CDCl_3$) δ 20.5; MS (FAB) *m/z* (rel intensity) 598 (MH^+, 8), 688 (9), 508 (5), 397 (5), 91 (100); HRMS (FAB) calcd for $C_{31}H_{40}N_3O_7PH$ (MH^+) 598.2682, found 598.2628. This material was used in the next step without further purification.

***O*-Benzyl-*O*-[3-[*N*-[4-[*N*-(benzyloxycarbonyl)amino]butyl]-*N*-(benzyloxycarbonyl)amino]propyl][[[(α-*O*-benzyl-*Z*-glutamyl)alanyl]amino]methyl]phosphonate (14).** To a stirred solution of **13** (61 mg, 0.14 mmol), **12** (82 mg, 0.14 mmol), and BOP reagent (67 mg, 0.15 mmol) in dry CH_2Cl_2 (8 mL) was added DIEA (54 mg, 0.41 mmol) slowly through a syringe at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and then at room temperature for 1.5 h. After removal of the volatile components, the residue was partitioned between EtOAc and H_2O (40/20 mL), the layers were separated, and the aqueous layer was extracted with EtOAc (2 × 20 mL). The combined EtOAc extracts were washed successively with 5% citric acid (2 × 30 mL), H_2O (30 mL), 5% $NaHCO_3$ solution (2 × 30 mL), H_2O (30 mL), and brine (40 mL) and then dried over Na_2SO_4. After removal of the solvent, the product was purified by column chromatography on silica gel eluting with a gradient of EtOAc to MeOH/EtOAc (1:9) to give 0.11 g (81%) of **14** as a colorless oil: ^1H NMR (DMSO-d_6) δ 1.14 (d, 3 H, $J = 7$), 1.25−1.38 (m, 2 H), 1.40−1.50 (m, 2 H), 1.70−1.86 (m, 3 H), 1.94−2.03 (br, 1 H), 2.22 (t, 2 H, $J = 1.7$), 2.94−3.02 (m, 2 H), 3.11−3.23 (m, 4 H), 3.54−3.67 (m, 2 H), 3.89−3.97 (m, 2 H), 4.06−4.14 (m, 1 H), 4.28−4.36 (m, 1 H), 4.95−5.14 (m, 10 H), 7.36 (m, 27 H), 7.80 (d, 1 H, $J = 6$), 8.04 (br, 1 H), 8.32 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 173.0, 172.6, 172.5, 156.5, 136.8, 136.4, 136.1, 136.7, 135.4, 128.8, 128.7, 128.4, 128.2, 127.9, 68.3, 68.2, 68.1, 67.4, 67.2, 66.8, 64.2, 63.7, 53.8, 49.0, 46.7, 43.9, 40.7, 35.8−34.1 (d, $^1J_{P-C} = 158.7$), 32.1, 29.1, 28.0, 27.2, 25.8, 25.4; ^{31}P NMR ($CDCl_3$) δ 23.8; MS (FAB) *m/z* (rel intensity) 1022 (MH^+, 1.3), 91 (100). Anal. ($C_{54}H_{64}N_5O_{13}P{\cdot}0.5H_2O$) C, H, N.

Hydrogen *O*-[3-[*N*-(4-Aminobutyl)amino]propyl][[(γ-glutamylalanyl)amino]methyl]phosphonate (4). A mixture of **13** (56 mg, 0.077 mmol) and 10% Pd/C (49 mg) in MeOH (15 mL) and AcOH (1 mL) was shaken under H_2 at 44 psi for 28 h. The catalyst was removed by filtration and the oily residue, obtained after the removal of MeOH, was dissolved in H_2O and purified by ion exchange chromatography (AG 50W-X8 cation exchange resin, NH_4^+ form) eluting with 0.08 M NH_4HCO_3 to afford 19.5 mg (83%) of **4** as a hygroscopic solid: R_f 0.32 (MeOH/AcOH (2:1), cellulose); $[\alpha]^{25}_D$ −22.8° (*c* 0.24, MeOH); ^1H NMR (D_2O) δ 1.36 (d, 3 H, $J = 7.4$), 1.70−1.81 (m, 4 H), 1.90−2.08 (m, 4 H), 2.40 (t, 2 H, $J = 8.8$), 2.95−3.12 (m, 6 H), 3.46 (d, 2 H, $J = 11.8$), 3.45−3.54 (m, 1 H), 3.9−3.98 (q, 2 H), 4.24−4.32 (q, 1 H); ^{13}C NMR (MeOH-d_4) δ 178.6, 176.1, 175.6, 63.6, 56.0, 51.0, 48.3, 46.4, 40.1, 36.8 (d, $J = 148.1$), 32.8, 29.5, 28.3, 25.8, 24.3, 17.8; ^{31}P NMR (D_2O) δ 18.5; MS (FAB) *m/z* (rel intensity) 440 (MH^+, 100), 309 (52); HRMS

(FAB) calcd for $C_{16}H_{34}N_5O_7PH$ (MH^+) 440.2274, found 440.2297. Anal. ($C_{16}H_{34}N_5O_7P{\cdot}H_2CO_3{\cdot}0.5H_2O$) C, H, N.

General Procedure for the Synthesis of Phosphonamidates. Method A.[16] Phosphonochloridate (1.0−1.4 mol equiv), prepared from the corresponding phosphonate monoester (**10** or **18**) and oxalyl chloride, was dissolved in dry CH_2Cl_2 (10−30 mL/mmol); TEA (1.2−2.4 mol equiv) and a catalytic amount of DMAP were added followed by the slow addition of the corresponding amine (1 mol equiv) at 0 °C. The reaction was continued at 0 °C for 30 min and at room temperature overnight. The reaction mixture was then worked up as described under method B.

Method B. To a stirred solution of the phosphonate monoester (**10** or **18**, 1 mol equiv), the amine **9** (1 mol equiv), and BOP reagent (1.2 mol equiv) in dry CH_2Cl_2 was added DIEA (2 mol equiv) dropwise at 0 °C. The reaction mixture was then allowed to stir at 0 °C for 10 min and at room temperature for 1.5 h. The volatile components were removed under reduced pressure, the residue was partitioned between EtOAc/H_2O (1:1, 60 mL), the EtOAc layer was separated, and the aqueous layer was extracted with EtOAc (2 × 30 mL). The combined EtOAc extracts were washed successively with 5% citric acid (2×), H_2O (1×), 5% $NaHCO_3$ solution (2×), H_2O (1×), and brine (1×), and dried over sodium sulfate. After removal of the solvent, the products were purified by recrystallization or column chromatography.

15a: mp 124−126 °C; R_f 0.62 (EtOAc); method A, 19%; method B, 100%; ^1H NMR ($CDCl_3$) δ 0.86 (t, 3 H, $J = 7$), 1.22−1.51 (m, 4 H), 2.86−3.10 (m, 3 H), 4.05−4.20 (m, 2 H), 5.10 (d, 2 H, $J = 7$), 7.16−7.43 (m, 5 H), 7.71−7.78 (m, 2 H), 7.80−7.89 (m, 2 H); ^{13}C NMR δ 166.5, 134.2, 132.1, 128.6, 128.3, 127.9, 124.9, 123.6, 120.5, 60.4, 40.6, 35.2 (d, $J = 127$), 34.3, 20.3, 14.2; ^{31}P NMR δ 24.4; MS (FAB) m/z (rel intensity) 387 (MH^+, 25), 259 (26), 219 (66), 145 (45), 136 (41), 91 (100); HRMS (FAB) calcd for $C_{20}H_{23}N_2O_4PH$ (MH^+) 387.1474, found 387.1468.

15b: oil; R_f 0.45 (EtOAc); method A, trace; method B, 73%; ^1H NMR ($CDCl_3$ + MeOH-d_4) δ 1.40−1.49 (overlap, 4 H), 1.51−1.73 (m, 2 H), 2.89−3.10 (br, 2 H), 3.10−3.78 (m, 6 H), 4.05−4.13 (m, 2 H), 5.06 (m, 6 H), 7.15−7.76 (m, 15 H), 7.68−7.75 (m, 2 H), 7.78−7.89 (m, 2 H); ^{13}C NMR δ 167.3, 157.0, 155.8, 136.4, 134.3, 131.7, 129.7, 128.4, 128.3, 128.0, 127.7, 127.6, 126.9, 123.4, 67.1, 66.4, 46.4 (46.1), 4.2, 37.8 (37.3), 40.3 (d, $J = 12$), 37.8 (37.3), 34.9 (d, $J = 143$), 30.7 (29.8), 26.8 (d, $J = 10$), 25.6 (25.0); ^{31}P NMR δ 24.7; MS (FAB) m/z (rel intensity) 727 (MH^+, 9), 511 (5), 307 (8), 153 (48), 91 (100); HRMS (DCI/CH_4) calcd for $C_{39}H_{43}N_4O_4PH$ 727.2897, found 727.2903.

19a: mp 113−115 °C; R_f 0.44 (MeOH/EtOAc (8:92)); method A, 70%; ^1H NMR ($CDCl_3$) δ 0.89 (t, 3 H, $J = 7.2$), 1.25−1.39 (m, 2 H), 1.40−1.54 (m, 2 H), 2.75−2.89 (m, 1 H), 2.90−3.06 (m, 2 H), 3.73 (d, 3 H, $J = 11$), 3.95−4.14 (m, 2 H), 7.70−7.80 (m, 2 H), 7.85−7.95 (m, 2 H); ^{13}C NMR δ 167.4, 134.3, 132.0, 123.6, 51.8, 40.6, 34.8 (d, $J = 141$), 34.4, 20.0, 13.9; ^{31}P NMR δ 25.3. Anal. ($C_{14}H_{19}N_2O_4P$) C, H, N.

19b: mp 57−59 °C; R_f 0.45 (EtOAc/TEA (4:1)); method A, 48%; method B, 61%; ^1H NMR ($CDCl_3$) δ 1.35−1.65 (overlap, 4 H), 1.65−1.75 (t, 2 H), 2.90−3.05 (overlap, 2 H), 3.10−3.42 (m, 7 H), 3.55−3.70 (m, 3 H), 3.75−4.05 (m, 2 H), 5.04 (s, 4 H), 7.15−7.40 (m, 10 H), 7.60−7.70 (m, 2 H), 7.75−7.85 (m, 2 H); ^{13}C NMR δ 167.0, 156.4, 136.5, 134.0, 131.6, 128.3, 128.2, 127.8, 127.5, 123.2, 66.8, 66.2, 51.2 (d, $J = 5.9$), 46.9, 46.2, 46.2, 43.9, 40.3, 37.8, 37.2, 34.5 (d, $J = 141$), 34.3 (d, $J = 141$), 30.9, 30.0, 26.9, 25.5, 25.0; ^{31}P NMR δ 25.5; MS (DCI/CH_4) m/z (rel intensity) 651 (MH^+, 11), 442 (31), 414 (93), 273 (100); HRMS (DCI/CH_4) calcd for $C_{33}H_{39}N_4O_8PH$ 651.2584, found 651.2581. Anal. ($C_{33}H_{39}N_4O_8P$) C, H, N.

General Procedure for the Synthesis of *O*-Alkyl-*N*-alkyl-[[[(Z-α-*O*-benzyl-γ-glutamyl)alanyl]amino]methyl]phosphonamidates. A mixture of phosphonamidate **15** or **19** (1 mol equiv) and $H_2NNH_2{\cdot}H_2O$ (10 mol equiv) in MeOH (ca. 10 mL/mmol) was stirred at room temperature for 48 h. The precipitate that formed was removed by filtration, and the filtrate was concentrated under reduced pressure. The resultant residue (white semisolid in most cases) was partitioned between CH_2Cl_2 and NH_4OH. CH_2Cl_2 layer was separated, and the aqueous layer was extracted with CH_2Cl_2. The organic extracts were combined and dried over Na_2SO_4. Removal of the solvent in vacuo afforded the corresponding (aminomethyl)phosphonamidate which was dissolved in dry CH_2Cl_2 (20 mL/mmol **15** or **19**). Dipeptide **13** (1 mol equiv) and BOP reagent (1.2 mol equiv) were then added followed by the slow addition of DIEA (2.0 mol equiv) at 0 °C. The reaction was continued at 0 °C for 10 min and then at room temperature for 1.5 h. After a standard extractive workup, the product was purified by recrystallization or column chromatography on silica gel. Crystalline products (**17**, **20a**) melt over a broad range due to the presence of diastereomeric mixtures.

17: mp 150−158 °C; 89% from **15a**; ^1H NMR ($CDCl_3$) δ 0.81−0.90 (q, 3 H), 1.25−1.40 (m, 7 H), 1.90−2.25 (m, 4 H), 2.75−2.95 (m, 2 H), 3.25−3.50 (m, 2 H), 3.75−3.86 (m, 1 H), 4.36−4.54 (m, 2 H), 4.90−5.00 (m, 1 H), 5.05−5.12 (m, 6 H), 6.30 (br d, 1 H), 6.65 (br d, 1 H), 7.15−7.35 (m, 15 H); ^{13}C NMR δ 173.0, 172.2, 172.1, 155.6, 136.4, 135.4, 128.8, 128.7, 128.5, 128.4, 127.9, 67.4, 67.2, 66.0, 53.9 (53.8), 49.4 (49.2), 36.8 (d, $J = 143$), 35.8 (d, $J = 143$), 34.1, 32.2, 28.2 (28.0), 19.9, 18.5 (18.2), 13.9; ^{31}P NMR δ 28.6, 28.5; MS (FAB) m/z (rel intensity) 681 (MH^+, 3), 608 (11), 154 (21), 91 (100); HRMS (FAB) calcd for $C_{35}H_{45}N_4O_8PH$ (MH) 681.3052, found 681.3064.

20a: mp 98−103 °C; 82% from **19a**; ^1H NMR ($CDCl_3$) δ 0.80−0.92 (m, 3 H), 1.15−1.45 (m, 7 H), 1.95−2.15 (dm, 2 H), 2.26−2.38 (m, 2 H), 2.65−2.88 (m, 2 H), 3.40−3.70 (m, 5 H), 4.05−4.35 (m, 1 H), 4.31−4.49 (m, 2 H), 4.99−5.12 (m, 5 H), 6.25−6.45 (m, 1 H), 7.05−7.45 (m, 10 H), 7.62−7.76 (br, 1 H); ^{13}C NMR δ 173.5, 172.6, 172.2, 156.6, 136.3, 135.4, 128.8, 128.6, 128.4, 128.3, 67.4, 67.2, 53.7, 50.9, 49.5, 40.6, 36.7 (d, $J = 143$) (35.5 (d, $J = 145$)), 34.0 (d, $J = 5$), 31.9, 27.9, 19.9, 18.1, 13.9; ^{31}P NMR δ 29.8; MS (FAB) m/z (rel intensity) 605 (MH^+, 4), 532 (19), 134 (9), 91 (100); HRMS (FAB) calcd for $C_{29}H_{41}N_4O_8PH$ (MH) 605.2740, found 605.2724.

20b: oil; R_f 0.5 (EtOAc/MeOH/TEA (16:4:1)); 91% from **19b**; ^1H NMR ($CDCl_3$) δ 1.31 (d, $J = 7$), 1.41−1.74 (overlap, 6 H), 2.05 (dm, 2 H), 2.20−2.29 (overlap, 2 H), 2.81−2.95 (overlap, 2 H), 3.05−3.31 (m, 6 H), 3.41−3.72 (overlap, 5 H), 3.80−4.02 (br, 1 H), 4.27−4.62 (overlap, 2 H), 4.95−5.10 (m, 8 H), 5.21−5.32 (br, 1 H), 6.50−6.63 (br, 1 H), 6.95−6.62 (br, 1 H), 7.15−7.45 (m, 20 H), 7.70−7.82 (br, 1 H); ^{13}C NMR δ 173.0, 172.1 (×2), 156.5, 156.0, 136.7, 136.3, 135.3, 128.5, 128.1, 127.7, 67.1, 66.8, 66.5, 53.8, 50.8, 48.9, 46.5, 44.4, 41.0 (40.6), 38.3 (37.7), 35.8 (d, $J = 142$), 32.0, 30.1, 27.6, 27.1, 25.7 (25.3), 18.4; ^{31}P NMR δ 29.2, 29.1, 28.9; MS (FAB) m/z (rel intensity) 945 (MH^+, 1.3), 793 (1.6), 523 (8.2), 414 (47), 91 (100); HRMS (FAB) calcd for $C_{48}H_{61}N_6O_{12}PH$ (MH^+) 945.4163, found 945.4199. Anal. ($C_{48}H_{61}N_6O_{12}P$) H, N; C: calcd, 60.98; found, 60.54.

***O*-Methyl-*N*-butyl[[(γ-glutamylalanyl)amino]methyl]phosphonamidate (21a).** *O*-Methyl-*N*-butyl[[[(Z-α-*O*-benzyl-γ-glutamyl)alanyl]amino]methyl]phosphonamidate (**20a**; 50 mg, 0.08 mmol) and 10% Pd/C (20 mg) in EtOH (15 mL) were shaken under H_2 (40 psi) on a Parr hydrogenator for 12 h, and the catalyst was then removed by filtration. The filtrate was concentrated to afford the product as a syrup which was triturated with ether to afford a white powder; 30 mg (95%) of pure **21a** was obtained by crystallization from EtOH/Et_2O: mp 178−189 °C dec (softens at 118 °C); ^1H NMR (MeOH-d_4) δ 0.87 (t, 3 H, $J = 7$), 1.21−1.48 (m, 7 H), 2.08−2.21 (m, 2 H), 2.47 (t, 2 H, $J = 6.5$), 2.81−2.94 (m, 2 H), 3.45−3.72 (m, 5 H), 3.86 (t, 1 H, $J = 6$), 4.20−4.39 (q, 1 H); ^{13}C NMR δ 175.3, 174.5, 172.6, 54.3, 51.8, 50.9, 41.5, 37.0 (d, $J = 144$), 35.6, 32.5, 27.4, 21.0, 18.0, 14.3; ^{31}P NMR δ 31.0; MS (FAB) m/z (rel intensity) 381 (MH^+, 100), 308 (94), 201 (12), 180 (21); HRMS (FAB) calcd for $C_{14}H_{29}N_4O_6PH$ (MH^+) 381.1903, found 381.1888.

***O*-Methyl-*N*-[3-[*N*-(4-aminobutyl)amino]propyl][[(γ-glutamylalanyl)amino]methyl]phosphonamidate (21b).** To a solution of **20b** (0.1 g, 0.1 mmol) in absolute EtOH (14 mL) was added a suspension of 10% Pd/C in EtOH (4 mL), and the mixture was shaken under H_2 (40 psi) on a Parr hydrogenator for 12 h. The catalyst was then removed by filtration, and the filtrate was concentrated under reduced pressure. The resultant residue was triturated with Et_2O/EtOH (20:1, v/v), and 38 mg (78%) of **21b** was obtained as a hygroscopic powder: R_f 0.64 (MeOH/AcOH/H_2O (4:1:1), cellulose); ^1H NMR (D_2O + MeOH-d_4) δ 1.34 (d, 3 H, $J = 7$), 1.62−1.75 (br, 4 H), 1.76−1.87 (overlap, 2 H), 2.04−2.10 (m, 2 H), 2.35−2.46 (t, 2 H, $J = 6$), 2.89−3.09 (overlap, 8 H), 3.51−3.70 (m, 6 H), 4.29−4.28 (q, 1 H); ^{13}C NMR δ 176.0, 175.6, 175.1, 55.3, 52.8, 51.0, 48.0, 46.2, 39.9, 38.3, 36.8 (d, $J = 143$), 32.2, 29.1, 27.8, 25.1, 23.9, 17.6; ^{31}P NMR δ 31.5, 31.4; MS (FAB) m/z (rel intensity) 453 (MH$^+$, 9), 202 (38), 180 (100); HRMS (FAB) calcd for $C_{17}H_{37}N_6O_6PH$ (MH) 453.2590, found 453.2575. All attempts to obtain an analytical sample of **21b** by use of ion exchange chromatography failed due to product instability during chromatography.

Enzyme Inhibition Assays. Inhibition of Gsp synthetase by the phosphonate **4**[21] and phosphonamidates **21a**,**b** was observed spectrophotometrically by coupling the hydrolysis of ATP to oxidation of NADH via pyruvate kinase/lactate dehydrogenase reactions.[20] The assay was initiated by adding purified Gsp synthetase (12.8 nM) to an assay mixture which contained the following components (final concentration): 1.56 mM glutathione, 10 mM spermidine, 2 mM ATP, 2.7 mM $MgCl_2$, 1 mM phospho(enol)pyruvate, 0.2 mM NADH, 50 μg/mL lactate dehydrogenase, 100 μg/mL pyruvate kinase, and various concentrations of inhibitor in 50 mM NaPIPES (pH 6.8) at 37 °C.

Noncompetitive or mixed-type inhibition was analyzed according to Scheme 4. Scheme 5 was used as the basis for the analysis of slow-binding inhibition. K_i and K_i^* are defined as shown in Scheme 5.[52]

The inhibition of Gsp amidase activity was assayed by coupling the production of EtOH (due to the hydrolysis of a substrate analog, glutathione ethyl ester) to the reduction of NAD through the activities of alcohol dehydrogenase and aldehyde dehydrogenase.[21] The assay mixture contained the following components: 1 mM NAD, 0.5 mg (170 units) of alcohol dehydrogenase, 0.07 mg (4 units) of aldehyde dehydrogenase, 1.28 nM purified Gsp amidase fragment, 2.5 mM GSH ethyl ester, and various concentrations of inhibitor in 50 mM NaPIPES (pH 6.8) at 37 °C.

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Supporting Information Available: Detailed procedures for the synthesis of **7** and **13**, including peptide precursors of **13** (2 pages). Ordering information is given on any current masthead page.

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