Design, Synthesis, and Activity of Analogues of Phosphinothricin as Inhibitors of Glutamine Synthetase

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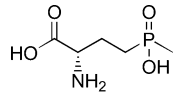
Received May 19, 2005

A new group of potent inhibitors of glutamine synthetase was designed and synthesized. The X-ray structure of bacterial glutamine synthetase complexed with phosphinothricin was used for computer-aided structure-based design of the inhibitors, in which the methyl group of phosphinothricin was chosen as the modification site. Amino and hydroxyl moieties were introduced into the phosphinic acid portion of the lead molecule to interact with ammonium binding site in the active cleft of the enzyme. Designed compounds were synthesized in enantiomerically pure form analogous to L-glutamic acid. In vitro kinetic studies with *Escherichia coli* glutamine synthetase confirmed the biological activity of the designed inhibitors, which with K_i values in the micromolar range ($K_i = 0.59$ μM for the most potent compound **2**) appear to be slightly weaker inhibitors or equipotent to phosphinothricin.

Introduction

Glutamine synthetase [GS; L-glutamate: ammonia ligase (ADP forming), EC 6.3.1.2] is a key enzyme in nitrogen metabolism of most living systems. It catalyses the first step in nitrogen assimilation, the ATP-dependent formation of glutamine from glutamate and ammonia.[1,2] The amide group is then transferred by a glutamate synthase (GOGAT) to α-ketoglutarate, yielding two glutamate molecules. The former closes the so-called GS−GOGAT cycle, the latter works in turn as a nitrogen donor in the biosynthesis of numerous biologically important compounds, including amino acids, purines, pyrimidines, and glucosamine.[3,4] GS-catalyzed reaction involves the initial synthesis of activated γ-glutamyl phosphate, followed by replacement of the phosphate group by ammonia with the formation of a phosphorylated tetrahedral intermediate.[5] X-ray analysis showed that bacterial GS is a homododecamer built of two hexameric rings of subunits, with 12 "bifunnel"-shaped active sites formed between monomers, where ATP and glutamate bind at opposite ends.[6] At the joint of each bifunnel, two bivalent cation-binding sites are located, to which either magnesium or manganese ions involved in phosphoryl transfer are bound.

The pivotal role of the enzyme in nitrogen metabolism stimulated research for GS inhibitors. Besides possible practical applications, the inhibitors may represent a tool for a better understanding of the catalytic properties, the regulative mechanisms, and the physiological role of the enzyme. The majority of both natural and synthetic GS inhibitors studied so far interact with the glutamate binding site, with phosphinothricin (PPT, **1**) and methionine sulfoximine being the most powerful (K_i of 0.6[7] and 1.0 μM[8] against *Escherichia coli* GS, respectively) and best known examples.[9−11]



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When bound to the target site, these compounds undergo phosphorylation in the presence of ATP.[12−15] Phosphorylated inhibitors mimic the tetrahedral intermediate and, presumably, the transition state of glutamine synthesis, resulting in irreversible, noncovalent inhibition of GS. Numerous compounds derived from the structure of PPT were synthesized and used to investigate the steric and electronic requirements needed for effective interaction with the glutamate binding site. Despite these efforts, in no case was the activity of the lead compound improved.[7,8,16−22] PPT and the natural tripeptide from which it originates (L-phosphinothricil-L-alanyl-L-alanine) have been successfully introduced to agricultural practice as nonselective herbicides because inactivation of plant GS causes a dramatic and rapid increase of toxic ammonia concentration inside the cell and subsequent death of the organism.[23]

A new promising perspective for GS inhibition studies is the design and synthesis of novel pharmaceuticals against *Mycobacterium tuberculosis*.[24−26] Tuberculosis (TB) is a leading cause of death due to a single infectious agent, with millions of new cases reported annually, no longer exclusively in underdeveloped countries.[27] New antibiotics are urgently needed to combat drug-resistant strains of *M. tuberculosis* and stop the emerging TB pandemics. Recently, GS has been identified as an important determinant of *M. tuberculosis* pathogenesis and thus a potential antibiotic target for TB treatment. A feature of mycobacteria is the extracellular release of numerous proteins, among which GS is abundant because of the involvement in the synthesis of poly-L-glutamate/glutamine complex required for proper formation of pathogen cell wall. Treatment with GS inhibitors strongly affects cell-surface polymer formation

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10.1021/jm050474e CCC: $30.25
Published on Web 09/14/2005

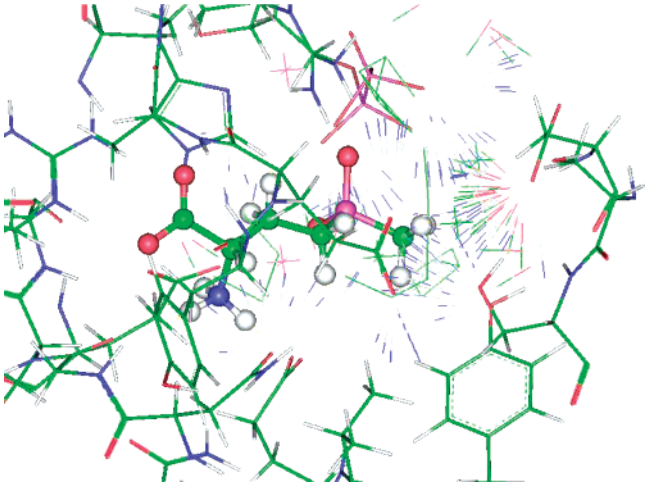


Figure 1. Structure of phosphinothricin−glutamine synthetase complex used for computer-assisted design of new PPT analogues. Interaction sites generated by LUDI are indicated as green (hydrophobic), green−red (acceptor of hydrogen bond), and blue−gray (donor of hydrogen bond) lines.

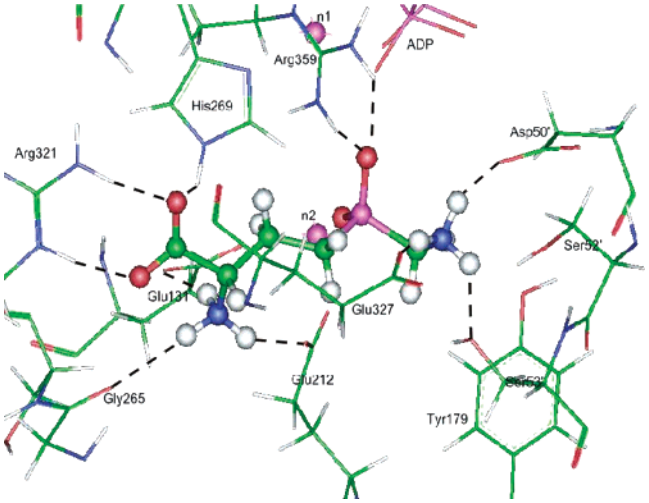


Figure 2. Binding pattern of compound **2** to glutamine synthetase. Enzyme residues involved in the interaction with the inhibitor are shown. Hydrogen bonds and interactions with metal ion are indicated as black dashed lines.

and selectively blocks the growth of several pathogenic mycobacteria, including *M. tuberculosis*, *Mycobacterium bovis,* and *Mycobacterium avia*, with no effect on host cells.[28−32] Therefore, the development of new potent inhibitors of glutamine synthetase is highly desirable as a possible therapeutic strategy against TB.

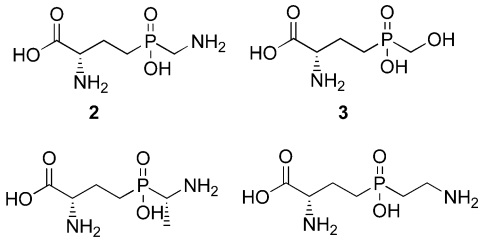
In this paper, we describe the computer-aided design of novel PPT derivatives based on the crystal structure of the complex formed by the inhibitor and bacterial GS.[33] A series of analogues of the lead compound with a modified methyl group was evaluated using LUDI program methodology, already successfully applied to the design of several potent inhibitors of other enzymes.[34,35] The most promising structures were synthesized in their enantiomerically pure form by means of multistep synthesis. Kinetic studies of the inhibition brought about by these compounds on *E. coli* GS proved the correctness of the applied computational methodology.

Results and Discussion

Computer-Aided Design. Phosphinothricin offers several advantages as a lead compound. It combines high inhibitory potency with a relatively simple structure that allows the introduction of a variety of modifications. Moreover, the crystal structure of PPT−GS complex was solved,[33] providing a basis for a rational computer-aided design. However, the conformation of PPT in the active site cannot be definitely determined from X-ray studies due to the isoelectronic structure of a methyl group and oxygen atom and the low resolution of the crystal structure. Extensive computations[36] indicated that the most probable arrangement of phosphinic moiety of PTT as that in which the methyl group is directed toward the ammonium binding site, while the oxygen atoms interact with Arg359 and n2 metal ion (Figure 1). Therefore, this structure was used for the computer-aided design of new inhibitors of glutamine synthetase.

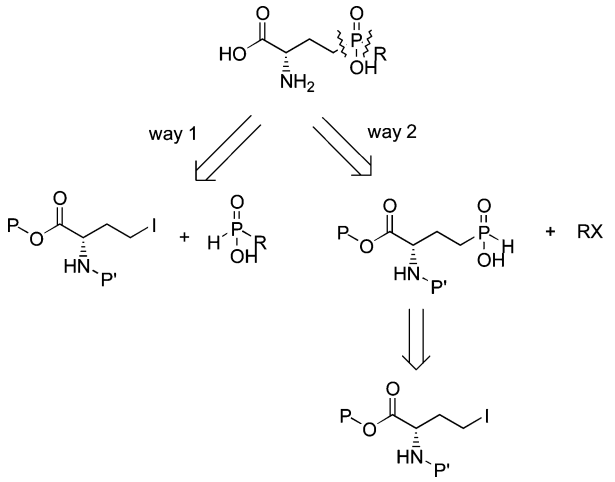
A preliminary analysis of PPT−GS complex showed that the inhibitor fits the active site quite well, and there is not much space for introducing new groups. However, it is still possible to add new small substituents for maximizing protein−inhibitor interactions. The most promising opportunity seemed to be the derivatization of the methyl group of PPT, as the only free space available is located around this moiety (Figure 1), with the biggest gap opened into the funnel near the binding site for the ATP triphosphate group. Most of protein surface surrounding the methyl moiety of PPT is negatively charged (residues Asp50′, Ser53′, Tyr179, Glu212, which under physiological conditions interact with ammonium ion[37]), suggesting that introduction of positively charged groups into the inhibitor structure might be favorable. Interaction sites generated by the program LUDI (Figure 1) indicated that, besides ionic forces, also hydrogen bonds may be of great importance, whereas hydrophobic interactions seem negligible.

LUDI from the Insight 2000 package (Accelrys) was used in Link_mode for the design of new PPT analogues, in which the methyl group was modified.[38−43] Among several propositions generated by the program, structures **2**−**5** were chosen as the most promising and characterized by high atomic economy.



The structures of inhibitor−enzyme complexes proposed by LUDI were additionally optimized using the cff97 force field[44] and then carefully inspected. The structure of the complex of inhibitor **2** with GS (Figure 2) showed that the former is tightly bound to the enzyme. Most interactions between **2** and GS were quite similar to those found for PPT, with ionic and hydrogen-bond interactions being the most important. The carboxylate group of the putative inhibitor forms hydrogen

Scheme 1



bonds with the guanidinium moiety of Arg321, and the α-amino group interacts with the γ-carboxylate groups of Glu131 and Glu212 and the oxygen atom of γ-amide of Asn264. One of the oxygen atoms of the phosphinic moiety interacts with n2 metal ion, while the second one forms two hydrogen bonds with Arg359. On the other hand, the introduction into PPT structure of the additional amino group located near the binding site of ammonium ion imposed new, strong hydrogen bonds between **2** and Asp50′ and Glu327 carboxylate groups.

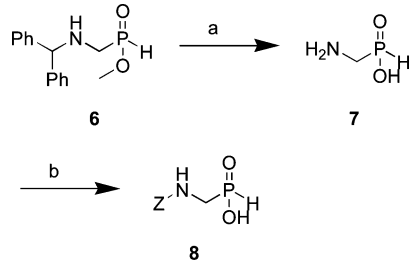
As predicted by our modeling studies, the mode of binding of the other designed compounds (**3**−**5**) was similar. The fragments of these inhibitors identical to PPT and **2** were found to interact with the enzyme in the same manner. Small differences were observed in the case of compound **3**, whose hydroxyl group can act as the donor of only one hydrogen bond (formed with Ser53′), and in the case of compound **5**, which due to steric reasons (a longer spacer between phosphorus and nitrogen atoms) also forms only one hydrogen bond (with Tyr179).

Chemistry

Although the synthesis of phosphorus-containing analogues of glutamic acid has been intensively studied,[45−53] it is not a trivial goal yet, particularly if aiming at the preparation of enantiomerically pure compounds. Thus, a retrosynthetic analysis was performed prior to the synthesis of the designed inhibitors (Scheme 1). The two most promising disconnections seemed those near the phosphorus atom. The first one would lead to the appropriate *H*-phosphinic acids and a suitable protected derivative of α-amino-γ-iodobutyric acid. The second one would suggest the use of phosphinic acid analogue of glutamate as the key substrate. Preliminary experiments ruled out the second possibility, as the synthesis of the phosphinic acid analogue, although having been published,[53] gave in our hands the desired product with a substantially low yield (below 5%). Because of these limitations, the first way of synthesis was applied.

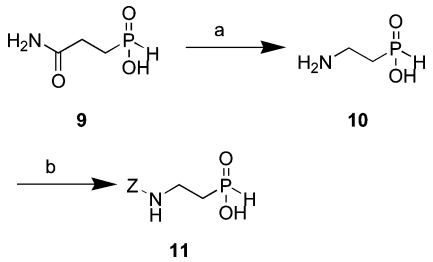
Starting substrate—methyl ester of (1*S*)-*N*-benzyloxycarbonyl-1-amino-3-iodobutyric acid **12**—was obtained from L-methionine by means of seven synthetic steps described in the literature.[54] At the same time, the synthesis of a series of *H*-phosphinic acids was performed. Thus, (1*R*)-*N*-benzyloxycarbonyl-1-aminoethylphosphinic[55] acid and hydroxymethylphosphinic[56] acid were obtained according to the literature. *N*-Benzyloxycarbonylaminomethylphosphinic acid was synthesized in three steps (Scheme 2). Acid hydrolysis of methyl ester **6**[57] yielded aminomethylphosphinic acid **7**, which was subsequently *N*-protected under standard basic conditions. To obtain the intermediate **10**, the Hoffman degradation of compound **9**[58] was applied (Scheme 3). The reaction was performed in a supercooled water solution in order to prevent side oxidation of phosphinic group to phosphonic one. The obtained 2-aminoethylphosphinic acid **10** was converted into its *N*-benzyloxycarbonyl derivative **11** using a standard procedure.[55]

Scheme 2[a]



[a] Reagents and conditions: (a) reflux, 8 h, concd HCl; (b) ZCl, NaOH, water/dioxane.

Scheme 3[a]

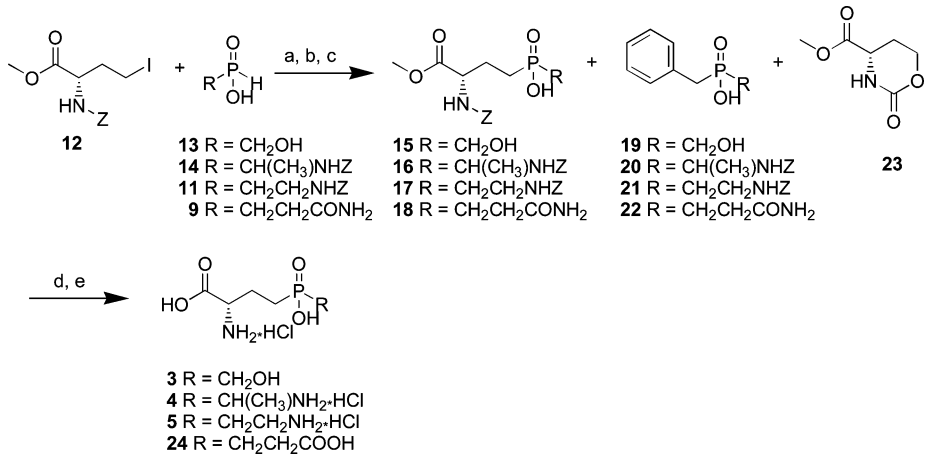


[a] Reagents and conditions: (a) OBr^-, −15 °C; (b) ZCl, NaOH, water/dioxane.

The main synthetic step was the reaction of the iodo derivative **12** with phosphinic acids (**9**, **11**, **13**, **14**). Phosphinic acids, preactivated by conversion into their trivalent trimethylsilyl diesters,[59] were allowed to react with compound **12** (Scheme 4) at 90 °C under inert atmosphere. Consequent methanolysis caused decomposition of the silyl derivatives, yielding the desired *N*-protected products **15**−**18**. These compounds were purified by HPLC. Unexpectedly, relatively large quantities of *P*-benzylalkanephosphinic acids (**19**−**22**) were also obtained. Since a similar reaction scheme upon the addition of the iodo derivative **12** to triethyl phosphite was already described,[45] the side-product formation might derive from an analogous mechanism (Scheme 5). Initially, compound **12** undergoes cyclization due to an intramolecular alkylation. Then, it can react with trivalent phosphorus compound, yielding *P*-benzylphosphinic acid and compound **23**. This route was additionally supported by isolation of compound **23** from the reaction mixture.

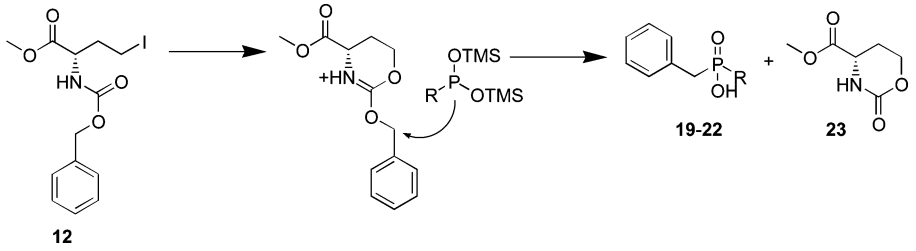
The reaction with the use of *N*-benzyloxycarbonyl-1-aminoethylphosphinic acid was performed with either racemic or enantiomerically pure substrate. In the first case, the obtained product was a mixture of two diastereomers (in 1:1 ratio), whereas in the second case, a single stereoisomer was present, as indicated by ^{31}P

Scheme 4[a]

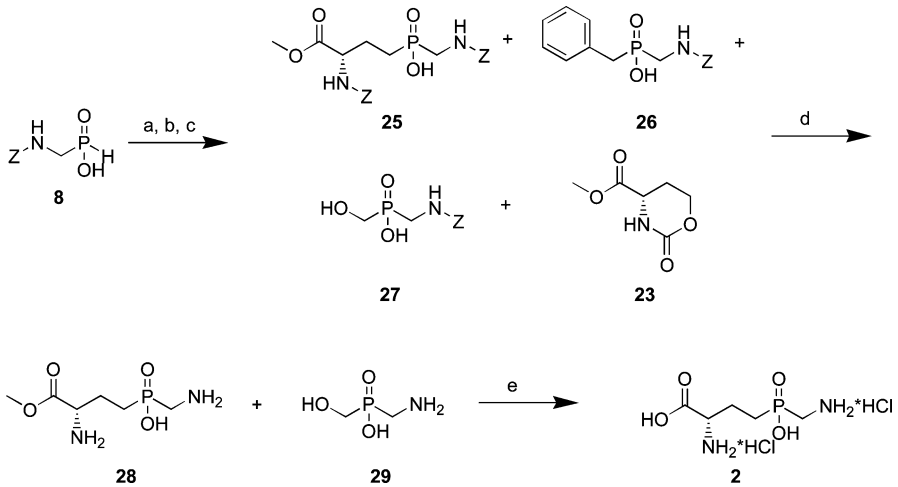


[a] Reagents and conditions: (a) hexamethyldisilazane, 90 °C, N_2; (b) heating, 90 °C, N_2; (c) MeOH; (d) HBr/AcOH or H_2, 10% Pd/C; (e) reflux, 8 h, concd HCl.

Scheme 5



Scheme 6[a]



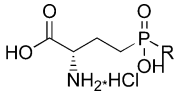
[a] Reagents and conditions: (a) hexamethyldisilazane, 90 °C, N_2; (b) compound **12,** 0 °C, then heating, 90 °C, 6 h, N_2; (c) MeOH; (d) H_2, 10% Pd/C; (e) reflux, 8 h, concd HCl.

NMR. This result confirmed the enantiomeric purity of both substrates as well as the corresponding product. Thus, consistently with literature data,[60] no racemization occurred under the applied reaction conditions.

Purified phosphinic acids **15**−**18** were finally deprotected in two subsequent synthetic steps. The benzyloxycarbonyl groups were removed using HBr in acetic acid at room temperature, and then methyl esters were hydrolyzed by refluxing in 20% aqueous hydrochloric acid, yielding the target inhibitors of satisfactory purity. The purity of final products was confirmed by HPLC using two different columns with reverse phase, namely Microsorb 300−10 C18 packing and ion-exchange Zorbax Sax packing. Only in the case of compound **25** did the deprotection and purification procedure have to be modified (Scheme 6) because, besides the expected side products (**23** and **26**), an unexpected side-product, compound **27**, was detected. Most likely it was formed upon reaction of silyl diester of phosphinic acid **8** with formaldehyde, resulting from partial thermal decomposition of this ester. Unfortunately, we did not succeed in its removal from the desired product **25** by means of HPLC. Thus, an additional chromatographic purification step, applied after partial deprotection by hydrogenolysis, was necessary to separate **28** from contaminating **29**. Finally, the pure methyl ester **28** was hydrolyzed, and the target product **2** was obtained in pure form.

Biological Activity of Phosphinothricin Analogues. The inhibitory potential of the obtained com-

Table 1. Inhibition of *E. coli* Glutamine Synthetase by Phosphinothricin (**1**) and Its Substituted Derivatives



compd	R	IC_{50} (μM)[a]	K_i (μM)[a]	pK_i
1	CH_3	1.2 ± 0.2[b]	0.6[c]	6.222
2	CH_2NH_2·HCl	1.0 ± 0.1	0.59 ± 0.05	6.229
3	CH_2OH	10.4 ± 1.7	2.1 ± 0.1	5.678
4	$CH(CH_3)NH_2$·HCl	31 ± 4	3.4 ± 0.6	5.469
(1′*RS*)-**4**	$CH(CH_3)NH_2$·HCl	33 ± 5	3.7 ± 0.2	5.432
5	$CH_2CH_2NH_2$·HCl	55 ± 8	13 ± 4	4.886
24	CH_2CH_2COOH	970 ± 180	150 ± 40	3.833

[a] Concentrations causing 50% inhibition (IC_{50}) of GS activity and inhibition constants against glutamate were evaluated as detailed in the Experimental Section. [b] Assuming inhibition by L-enatiomer only. [c] According to literature.[7]

pounds toward *E. coli* GS was assessed under conditions in which the enzyme catalyses the physiological, full biosynthetic reaction. All compounds showed strong inhibitory activity at micromolar levels (Table 1), as documented by both K_i and IC_{50} values. As expected, compound **2** was the most potent among studied inhibitors with an inhibition constant (K_i) of 0.59 μM. The high inhibitory activity of **2** most likely results from interactions of the additional amino group with residues near the ammonium ion binding site. This was confirmed by kinetics studies, which showed that compound **2** exerts an inhibition of competitive type against glutamate and uncompetitive type against ammonium ion and ATP (Figure 3). Thus, the glutamate-shared portion of compound **2** binds as glutamate itself, whereas the introduced amino group binds closely, but not exactly, to the ammonium ion binding site. The mode of action of **2** was further investigated by assaying the recovery of GS activity following pretreatment with either this compound or PPT and their removal by means of ammonium sulfate precipitation. As expected, in the case of PPT, a significant loss of activity was found as a consequence of the treatment ($48.8 \pm 3.0\%$ of the control, $n = 3$). On the contrary, after incubation with compound **2**, enzyme activity was fully recovered ($112.4 \pm 7.0\%$). Reversible inhibition indicated that the inhibitory mechanism of compound **2** differs from that of PPT. Either it does not undergo phosphorylation in the active site of the enzyme or (taking into account its close structural resemblance to PPT) the product of compound **2** phosphorylation is unstable under the employed assay conditions. Compound **2** might be considered as either an expanded transition state analogue of reaction between ammonia and glutamyl phosphate or a "collected substrate" analogue (analogue combining entities of both substrates, glutamate and ammonium ion). In the latter case, its amino group mimics ammonium ion, whereas phosphinic moiety mimics the tetrahedral intermediate of the reaction. Because the two groups are connected by a short and nonflexible methylene spacer, and a limited space is available in the active site, it is easy to understand why this inhibitor is not much more active than the lead PPT. Because of the reversibility of its action, we assume that it rather acts as a "collected substrate" analogue.

Replacement of amino moiety of compound **2** by hydroxyl group yielded compound **3**. This was accompanied by a significant loss of inhibitory activity (Table 1). Protonated amino group NH_3^+ not only is able to form a more complex network of hydrogen bonds than a hydroxylic one but, due to the positive charge, the group also more effectively interacts with negatively charged residues of the active site involved in ammonium ion binding.

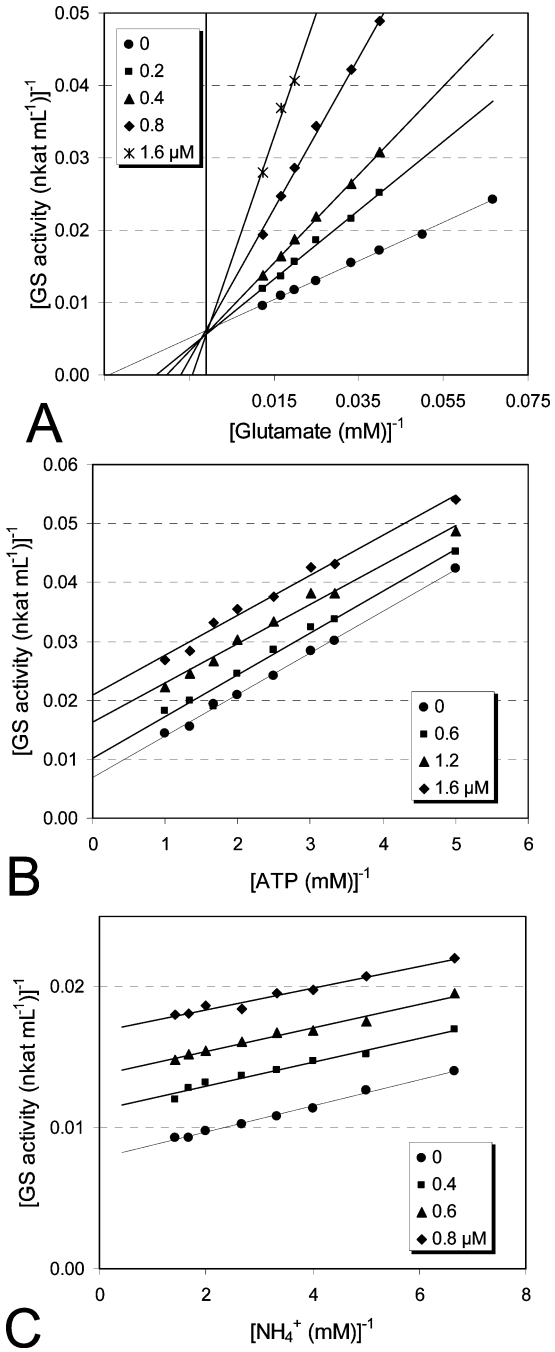


Figure 3. Kinetic analysis of GS inhibition by compound **2**. Lineweaver−Burk plots obtained at varying glutamate (A), ATP (B), and ammonia (C) concentrations in the presence of micromolar levels of the inhibitor accounted for an inhibition of competitive type against glutamate ($K_i = 0.59 \pm 0.05$ μM) and uncompetitive type with respect to either ATP and ammonia ($K_i = 0.84 \pm 0.06$ μM and 0.73 ± 0.04 μM, respectively).

Compound **4** was synthesized either as a single isomer (required from computer-aided design) or as mixture of diastereoisomers (1′*RS*)-**4**. Quite surprisingly, the two forms showed nearly identical binding affinities, indi-

cating that both stereoisomers (1′*R*)-**4** and (1′*S*)-**4** interact with the enzyme with comparable energy. It is worth noting that the negative steric effect of an additional methyl substituent in case of compound **4** (if compared with compound **2**) and the negative electronic effect of exchanging $-NH_3^+$ for $-OH$ group in compound **3** gave similar effects from a quantitative point of view, since the inhibition constants of compounds **3** and **4** were found to be close to each other.

Compound **5** is a homologue of compound **2** in which the methylene bridge between ammonium and phosphinate group was replaced by an ethylene fragment. This resulted in a significant loss of inhibitory activity (Table 1), once more indicating an unusual sensitivity toward small modification of the phosphinic fragment of PPT. As shown by molecular modeling, a longer bridge causes the extension of the amino group beyond the ammonium ion binding site. This results in the reduction of positive interactions between this group and the enzyme and leads to the formation of only one favorable hydrogen bond.

The low activity exerted by compound **24**, in which a negatively charged carboxylic group replaced the amino group of compound **5**, is consistent with computational results that suggest that substituents of the methyl group of PPT have to be positively charged rather than being donors of hydrogen bonds. A carboxylic group is negatively charged under physiological conditions, and this causes repulsive interactions between negatively charged portions of the enzyme. Such a result also clearly shows that proposals to incorporate negatively charged groups (for example phosphonic or phosphinic) attached to the methyl moiety of PPT[21,61] in order to mimic phosphorylated glutamate are incorrect.

Conclusions

A group of low molecular weight, potent inhibitors of bacterial GS was designed using molecular modeling techniques based upon the crystal structure of phosphinothricin−glutamine synthetase complex. The methyl group of the lead compound was substituted with small hydrophilic groups able to interact with the enzyme by means of electrostatic forces or hydrogen bonds. Introduced amino or hydroxyl moieties dock in the active cleft of the target enzyme near the ammonium ion binding site. All obtained analogues exerted exceptionally high inhibitory effect upon the physiological GS-catalyzed reaction. Substitution of PPT methyl group with amino moiety (compound **2**) proved to be the most effective, resulting in a K_i value as low as 0.59 μM. Experimental data obtained with *E. coli* GS inhibition studies were in qualitative agreement with LUDI computational predictions. As the inhibition by **2** occurred to be reversible, this compound most probably acts as "collected substrate" rather than transition-state inhibitor of GS. Comparison of the action of this compound with that of other designed PPT derivatives clearly showed that the enzyme is extremely sensitive to small structural changes introduced into the inhibitor molecule. Nevertheless, the efficacy of all designed inhibitors confirms the correctness of the proposed conformation of PPT in GS active site, as well as the usefulness of the applied computer-aided methodology.

Experimental Section

Chemistry. General. Materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck) and used without purification, unless otherwise stated. Triethylamine was distilled and stored over potassium hydroxide pellets. Column chromatography was performed on silica gel 60 (70−230 mesh). High performance liquid chromatography (HPLC) was carried out using a ProStar 210 apparatus from Varian Ltd. Preparative samples were injected onto a Dynamax 250 mm × 21.4 mm column with Microsorb 300−10 C18, with monitoring of the eluate at both 210 and 254 nm. Water purified with EASY PURE II Ultrapure Water Systems apparatus (Barnstead Ltd) and HPLC-grade acetonitrile (POCh, Gliwice, Poland) were used as eluents. Solution A was prepared as aqueous 1% trifluoroacetic acid and solution B was 1% trifluoroacetic acid in acetonitrile. Analytical samples were injected onto a Dynamax 250 mm × 4.6 mm column with Microsorb 300−10 C18 and eluted with 1% TFA in water and onto an Agilent, Zorbax Sax 150 mm × 4.6 mm column and eluted with 0.2 M phosphate buffer (pH=6.0).

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker Avance DRX 300 spectrometer operating at 300.13 MHz for ^{1}H, 75.48 MHz for ^{13}C, and 121.50 MHz for ^{31}P. Measurements were made in $CDCl_3$ (99.5% D, stabilized with silver) or D_2O (99.8%D) obtained from ARMAR Chemicals AG (Dötingen, Switzerland). Proton and carbon chemical shifts are reported in relation to tetramethylsilane used as internal standard. ^{31}P NMR spectra were recorded with use of broadband ^{1}H decoupling, and chemical shifts are reported in relation to 85% H_3PO_4 used as external standard.

Methyl *N*-Diphenylmethylaminomethylphosphinate (6). The compound was synthesized using a previously described procedure.[57] ^{1}H NMR ($CDCl_3$): δ 2.95 (d, J = 11.4 Hz, 3H, C*H*$_3$), 3.75 (d, J = 12 Hz, 2H, C*H*$_2$P), 4.84 (s, 1H, Ph$_2$C*H*), 7.06 (d, J = 549 Hz, 1H, P*H*), 7.11−7.37 (m, 10H, 2 × C$_6$*H*$_5$). ^{31}P NMR ($CDCl_3$): δ 39.1.

Aminomethylphosphinate Hydrochloride (7). Concentrated hydrochloric acid (100 mL) was added to compound **6** (9.6 g, 35 mmol), and the mixture was refluxed for 12 h. Then acid was removed under reduced pressure. Water (30 mL) and diethyl ether (30 mL) were added to the residue. Obtained mixture was filtered in order to remove solid particles and the organic layer was discarded. The water layer was washed with diethyl ether (2 × 20 mL) and evaporated under reduced pressure to yield 4.0 g (87%). ^{1}H NMR (D_2O): δ 2.84 (d, J = 16.6 Hz, 2H, C*H*$_2$), 6.98 (d, J = 549 Hz, 1H, P*H*). ^{31}P NMR (D_2O): δ 16.7.

***N*-Benzyloxycarbonylaminomethylphosphinic Acid (8).** Compound **7** (3.0 g, 23 mmol) was dissolved in 40 mL of water, and 4 M NaOH aqueous solution was added until pH 10 was reached. Then dioxane (20 mL) and benzyl chloroformate (4.3 mL, 30 mmol) were added. Mixture was stirred for 3 h while pH 10 was maintained, and then the solution was left overnight. Solvents were evaporated under reduced pressure, and water (50 mL) was added to the oily residue. The obtained solution was washed with diethyl ether (30 mL) and acidified with 2 M HCl to pH 2. Product was extracted with ethyl acetate (3 × 50 mL). Collected organic layers were dried over magnesium sulfate and evaporated, yielding 3.0 g of product of satisfactory purity (56%). ^{1}H NMR (D_2O): δ 3.10 (d, J = 9.8 Hz, 2H, C*H*$_2$P), 4.98 (s, 2H, PhC*H*$_2$), 6.79 (d, J = 523 Hz, 1H, P*H*), 7.14−7.29 (m, 5H, C$_6$*H*$_5$). ^{31}P NMR (D_2O): δ 22.9.

2-Aminoethylphosphinic Acid (10). NaOH (5.0 g, 125 mmol) was dissolved in water (50 mL) and cooled to 0 °C, then bromine (1.45 mL, 28 mmol) was added, and mixture was cooled to −15 °C. Compound **9**[58] (3.9 g, 29.5 mmol) was dissolved in water (5 mL) and solution was cooled to −15 °C and added to stirred hypobromine solution. The mixture was stirred at −15 °C for 2 h and then left overnight. 3 M HCl was added to the reaction mixture in order to reach pH 1 and the solution was evaporated under reduced pressure. The obtained residue was treated with ethanol (100 mL) and the solid material filtered off. Propylene oxide was added dropwise to remove hydrogen chloride and left overnight in a

refrigerator. Precipitated product was collected by filtration to yield 2.5 g (77%). ^{1}H NMR (D_2O): δ 1.82 (dt, J_{PH} = 11.3 Hz, J_{HH} = 6.6 Hz, 2H, CH_2P), 3.06 (dt, J_{PH} = 9.2 Hz, J_{HH} = 7.4 Hz, 2H, CH_2N), 6.97 (d, J = 546 Hz, 1H, P*H*). ^{31}P NMR (D_2O): δ 25.7.

***N*-Benzyloxycarbonyl-2-aminoethylphosphinic Acid (11).** The compound was obtained from **10** (1.6 g, 14 mmol) and benzyl chloroformate (2.7 mL, 19 mmol) using the same procedure as for compound **8** to yield 1.85 g (54%). ^{1}H NMR (D_2O): δ 1.61 (dt, J_{PH} = 15 Hz, J_{HH} = 7.3 Hz, 2H, CH_2P), 3.17 (dt, J_{PH} = 11.2 Hz, J_{HH} = 8.0 Hz, 2H, CH_2N), 4.95 (s, 2H, PhCH_2), 6.82 (d, J_{PH} = 510 Hz, 1H, P*H*), 7.27 (m, 5H, C_6H_5). ^{31}P NMR (D_2O): δ 27.2.

Methyl (2*S*)-2-Benzyloxycarbonylamino-4-(hydroxymethyl(hydroxy)phosphino)butyrate (15). Hexamethyldisilazane (7.0 mL) was added to hydroxymethylphosphinic acid[56] (0.25 g, 2.5 mmol) and the mixture was heated at 90 °C for 2 h in nitrogen atmosphere. Then solution was cooled to 0 °C and compound **12** (0.37 g, 1.0 mmol) was added. The mixture was heated at 90 °C for 6 h, and after cooling to room temperature, methanol (30 mL) was added and stirring was continued for 1 h. Solid impurities were filtered off, and the solution was evaporated under reduced pressure. Products were purified using HPLC (water/acetonitrile) to yield 60 mg of compound **15** (17%), 15 mg of compound **19** (8%), and 10 mg of compound **23** (6%). Compound **15**. ^{1}H NMR (D_2O): δ 1.65 (m, 2H, CH_2CH_2P), 1.76, 1.95 (m each, 1H and 1H, CHCH_2), 3.57 (s, 3H, OCH_3), 3.61 (d, J = 5.2 Hz, CH_2OH), 4.10 (t, J = 5.3 Hz, 1H, C*H*), 4.96 (s, 2H, PhCH_2), 7.24−7.27 (m, 5H, C_6H_5). ^{31}P NMR (D_2O): δ 49.8.

***P*-Benzylhydroxymethylphosphinic Acid (19).** ^{1}H NMR (D_2O): δ 3.05 (d, J = 16.6 Hz, 2H, PhCH_2), 3.55 (d, J = 5.2 Hz, 2H, CH_2OH), 7.14−7.24 (m, 5H, C_6H_5). ^{31}P NMR (D_2O): δ 46.2.

Methyl 2-Aza-4-oxa-3-oxocyclohexanecarboxylate (23). ^{1}H NMR (D_2O): δ 2.14−2.22, 2.32−2.40 (m each, 1H and 1H, CHCH_2), 3.16 (t, J = 7.1 Hz, 2H, CH_2CH_2O), 4.68 (s, 3H, OCH_3), 4.07 (t, J = 6.6 Hz, 1H, C*H*).

Methyl (2*S*)-2-Benzyloxycarbonylamino-4-[(1′-benzyloxycarbonylaminoethyl)(hydroxy)phosphinyl]butyrate (16). The compound was synthesized in the same way as **15** using compound **12** (0.37 g, 1.0 mmol) and *N*-benzyloxycarbonyl-1-aminoethylphosphinic acid (0.40 g, 1.6 mmol) to yield 45 mg (9%) of compound **16** and 25 mg (7%) of compound **20**. Compound **16**. ^{1}H NMR (D_2O): δ 1.30 (dd, J_{PH} = 14.6 Hz, J_{HH} = 7.0 Hz, 3H, CHCH_3), 1.75 (m, 2H, CH_2P), 1.85, 2.15 (m each, 1H and 1H, CHCH_2), 3.69 (s, 3H, OCH_3), 4.03, 4.32 (m each, 1H and 1H, 2 × C*H*), 4.91−5.11 (m, 4H, 2 × PhCH_2), 5.62 (dd, J_{PH} = 19.9 Hz, J_{HH} = 9.1 Hz, 1H, PCHN*H*), 5.94 (d, J = 8.7 Hz, 1H, CH_2CHNH), 7.24−7.32 (m, 10H, 2 × C_6H_5). ^{31}P NMR (D_2O): δ 56.04, 56.48 (two diastereomers, molar ratio 1:1).

***P*-Benzyl-1-benzyloxycarbonylaminoethylphosphinic Acid (20).** ^{1}H NMR (D_2O): δ 1.15 (dd, J_{PH} = 14.5 Hz, J_{HH} = 7.2 Hz, 3H, CHCH_3), 2.99 (d, J = 15.9 Hz, 2H, PhCH_2P), 3.96 (dt, J = 7.7 Hz, J = 5.5 Hz, 1H, C*H*), 5.07 (s, 2H, PhCH_2O), 5.11 (m, 1H, N*H*), 7.18−7.32 (m, 10H, 2 × C_6H_5). ^{31}P NMR (D_2O): δ 52.30 (90%) and 51.40 (10%) (trans and cis isomers).[62]

Methyl (2*S*)-2-Benzyloxycarbonylamino-4-[(2′-benzyloxycarbonylaminoethyl) (hydroxy)phosphinyl]butyrate (17). The compound was synthesized in the same way as **15** using compound **12** (0.37 g, 1.0 mmol) and compound **11** (0.49 g, 2.0 mmol) to yield 40 mg (8%) of compound **17** and 25 mg (7%) of compound **21**. Compound **17**. ^{1}H NMR (D_2O): δ 1.42 (m, 2H, $CHCH_2CH_2P$), 1.65, 1.84 (m each, 1H and 1H, CHCH_2), 1.65 (m, 2H, $NHCH_2CH_2P$), 3.16 (dt, J_{HH} = 8.3 Hz, J_{PH} = 7.3 Hz, 2H, CH_2N), 3.25 (s, 3H, OCH_3), 3.85 (m, 1H, C*H*), 5.00, 5.03 (s each, 2H and 2H, 2 × PhCH_2O), 7.31 (m, 10H, 2 × C_6H_5). ^{31}P NMR (D_2O): δ 43.0.

***P*-Benzyl-2-benzyloxycarbonylaminoethylphosphinic Acid (21).** ^{1}H NMR (D_2O): δ 1.49 (m, 2H, CH_2CH_2P), 2.75 (d, J = 17.2 Hz, PhCH_2P), 3.03 (m, 2H, CH_2CH_2P), 4.90 (s, 2H, PhCH_2O), 7.07−7.27 (m, 5H, C_6H_5). ^{31}P NMR (D_2O): δ 39.9.

Methyl (2*S*)-2-Benzyloxycarbonylamino-4-[(2′-carbamoylethyl)(hydroxy)phosphinyl]butyrate (18). The compound was synthesized in the same manner as **15** using compound **12** (0.37 g, 1.0 mmol) and carbamoylethylphosphinic acid[58] (0.28 g, 2.0 mmol) to yield 70 mg (18%). ^{1}H NMR (D_2O): δ 1.63−1.96 (m, 6H, $CHCH_2CH_2PCH_2$), 2.45 (m, 2H, CH_2-$CONH_2$) 3.60 (s, OCH_3), 4.15 (t, J = 5.4 Hz, C*H*), 4.98 (s, 2H, PhCH_2O), 7.28 (m, 5H, C_6H_5). ^{31}P NMR (D_2O): δ 57.15.

Methyl (2*S*)-2-Amino-4-[(aminomethyl)(hydroxy)phosphinyl]butyrate (28). Compound **8** (0.46 g, 2.0 mmol) and hexamethyldisilazane (7.0 mL) were heated at 90 °C for 1 h under inert nitrogen atmosphere. Then, the solution was cooled to 0 °C and compound **12** (0.37 g, 1.0 mmol) was added. Mixture was heated at 80 °C during 6 h. After cooling to room temperature, methanol (30 mL) was added and solution was additionally stirred for 1 h. Solid impurities were filtered off, and the solution was evaporated under reduced pressure. A mixture of compounds **25** and **27** was isolated using HPLC (water/acetonitrile). The obtained compounds were dissolved in methanol (10 mL) and hydrogenated over palladium catalyst (10% Pd/C, 30 mg) during 2 h. The catalyst was filtered off and filtrate evaporated. Residue was separated using HPLC (water/acetonitrile) to yield 5.3 mg (1.2%) of compound **28** and 4.0 mg (1.6%) of compound **29**. Compound **28**. ^{1}H NMR (D_2O): δ 1.66 (m, 2H, $CHCH_2CH_2P$), 2.09 (m, 2H, CHCH_2), 2.97 (d, J = 9.3 Hz, 2H, NH_2CH_2P), 3.75 (s, 3H, OCH_3), 4.12 (t, J = 6.3 Hz, 1H, C*H*). ^{31}P NMR (D_2O): δ 32.02.

Aminomethyl(hydroxymethyl)phosphinic Acid (29). ^{1}H NMR (D_2O): δ 3.12 (d, J = 10.2 Hz, 2H, PCH_2NH_2), 3.33 (d, J = 9.4 Hz, 2H, PCH_2OH). ^{31}P NMR (D_2O): δ 20.07.

(2*S*)-2-Amino-4-[(hydroxymethyl)(hydroxy)phosphinyl]-butyric Acid (3). Compound **15** (60 mg) was dissolved in methanol (10 mL) and hydrogenated over palladium catalyst (10% Pd/C, 10 mg) for 3 h. The catalyst was filtered off and filtrate evaporated. Dry residue was dissolved in concentrated HCl (10 mL) and refluxed for 8 h. Solvent was evaporated under reduced pressure and the product was purified by means of HPLC (water/acetonitrile) to yield 38 mg (94%). ^{1}H NMR (D_2O): δ 1.84 (m, 2H, $CHCH_2CH_2P$), 2.11 (m, 2H, CHCH_2-CH_2P), 3.75 (d, J = 5.0 Hz, 2H, CH_2OH), 4.07 (t, J = 5.0 Hz, 1H, C*H*). ^{31}P NMR (D_2O): δ 46.2. ^{13}C NMR (D_2O): δ 21.60 (d, J_{PC} = 89.5 Hz, CH_2CH_2P), 22.20 ($CHCH_2$), 52.82 (d, J_{PC} = 15.4 Hz, *C*H), 58.05 (d, J_{PC} = 110.6 Hz, PCH_2OH), 170.96 (*C*OOH). HPLC purity >97%.

(2*S*)-2-Amino-4-[(1′-aminoethyl)(hydroxy)phosphinyl]-butyric Acid (4). A solution of 30% HBr in acetic acid (3 mL) was added to compound **16** (40 mg) and the mixture was stirred for 12 h at room temperature. Then solution was evaporated under reduced pressure. Dry residue was dissolved in concentrated HCl (10 mL) and refluxed for 8 h. Solvent was evaporated under reduced pressure and the product was purified by means of HPLC (water/acetonitrile) to yield 21 mg (88%). ^{1}H NMR (D_2O): δ 1.35 (dd, J_{PH} = 12.0 Hz, J_{HH} = 6.4 Hz, 3H, CHCH_3), 1.72 (m, 2H, CH_2P), 2.14 (m, 2H, CHCH_2-CH_2), 3.31 (m, 1H, C*H*CH_3), 4.04 (t, J = 6.3 Hz, 1H, C*H*CH_2). ^{31}P NMR (D_2O): δ 36.01. ^{13}C NMR (D_2O): δ 12.80 (*C*H_3), 22.76 ($CHCH_2$), 23.15 (d, J_{PC} = 93.8 Hz, CH_2CH_2P), 45.77 (d, J_{PC} = 94.9 Hz, *C*HP), 53.60 (*C*HCOOH), 172.02 (*C*OOH). HPLC purity >97%.

(2*S*)-2-Amino-4-[(2′-Aminoethyl)(hydroxy)phosphinyl]-butyric Acid (5). The compound was synthesized from **17** (40 mg) using the same procedure as for **4** to yield 15 mg (65%). ^{1}H NMR (D_2O): δ 1.78 (m, 2H, $CHCH_2CH_2P$), 2.06 (m, 4H, $NCH_2CH_2PCH_2CH_2CH$), 3.17 (dt, J_{PH} = 7.2 Hz, J_{HH} = 8.4 Hz, 2H, CH_2NH_2), 4.10 (t, J = 5.7 Hz, 1H, C*H*). ^{31}P NMR (D_2O): δ 47.3. ^{13}C NMR (D_2O): δ 22.45 ($CHCH_2$), 24.60 (d, J_{PC} = 93.0 Hz, $CHCH_2CH_2P$), 25.67 (d, J_{PC} = 90.4 Hz, NCH_2CH_2P), 33.87 (CH_2CH_2N), 52.88 (d, J_{PC} = 16.0 Hz, *C*HCH_2), 171.06 (*C*OOH). HPLC purity >97%.

(2*S*)-2-Amino-4-[(2′-Carboxyethyl)(hydroxy)phosphinyl]-butyric Acid (24). The compound was synthesized from **18** (70 mg) using the same procedure as for **4** to yield 47 mg (95%). ^{1}H NMR (D_2O): δ 1.84 (m, 2H, $CHCH_2CH_2P$), 2.03 (dt, J_{PH} = 13.2 Hz, J_{HH} = 8.0 Hz, 2H, PCH_2CH_2COOH), 2.12 (m, 2H,

$CHCH_2CH_2P$), 2.54 (dt, J_{PH} = 12.6 Hz, J_{HH} = 7.5 Hz, 2H, PCH_2CH_2COOH), 4.09 (t, J = 6.0 Hz, 1H, CH). ^{31}P NMR (D_2O): δ 54.8. ^{13}C NMR (D_2O): δ 22.36 ($CHCH_2$), 23.13, 23.83 (d each, J_{PC} = 93.2 Hz and J_{PC} = 91.6 Hz, CH_2PCH_2), 26.25 (CH_2COOH), 52.80 (d, J_{PC} = 16.4 Hz, CH), 170.96 ($CHCOOH$), 176.32 (d, J = 14.1 Hz, CH_2COOH). ESI-MS (m/z): 240 (M + 1). HPLC purity >97%.

(2*S*)-2-Amino-4-[(aminomethyl)(hydroxy)phosphinyl]-butyric Acid (2). Compound **28** (3.3 mg, 0.012 mmol) was dissolved in concentrated HCl (10 mL) and refluxed for 8 h. Then solvent was evaporated under reduced pressure yielding 3.0 mg (95%) of the desired product of good purity. ^1H NMR (D_2O): δ 1.73 (m, 2H, $CHCH_2CH_2P$), 2.07 (m, 2H, $CHCH_2$), 2.98 (d, J = 9.3 Hz, 2H, NH_2CH_2P), 4.00 (t, J = 6.1 Hz, 1H, CH). ^{31}P NMR (D_2O): δ 33.53. ^{13}C NMR (D_2O): δ 22.90 ($CHCH_2$), 25.06 (d, J_{PC} = 96.0 Hz, $CHCH_2CH_2P$), 37.37 (d, J_{PC} = 91.8 Hz, PCH_2NH_2), 53.47 (d, J_{PC} = 15.1 Hz, CH), 171.85 ($COOH$). HPLC purity >97%.

Computations. The crystal structure of phosphinothricin−glutamine synthetase complex refined to 2.49 Å obtained from Protein Data Bank (1FPY) was used as the starting point for all calculations.[33] The hydrogen atoms were added using Insight 2000 (Accelrys).[63] The protonation states of the amino acid side chain residues were set up for pH 7.0. Manganium ion atoms were replaced with magnesium, due to the lack of parameters for manganium in the force field used (this is in agreement with biochemical data proving that exchange does not substantially change activity of the enzyme).[1] The LUDI module of Insight 2000 was used for designing GS inhibitors.[64] A search of the LUDI fragment library containing about 1000 structural fragments was performed. The search was done in the Link_mode of LUDI. The structures of inhibitors were obtained by substitution of methyl group of PPT with new structural groups generated by the computer program. Proposed groups were assumed to interact with the protein near the ammonium ion binding place. The main parameters of the LUDI program were set as follows: Max_Aligment_Angle = 14; Max_RMS = 0.4−0.6; Rotable_Bonds = Two_at_a_time; Radius = 6−10; Min_Separation = 3.0; Electrostatic_Check = On; Link_Weight = 1.0; Lipo_Weight = 1.0; H_Bond_Weight = 1.0; No_Unpaired_Polar = Yes; Reject_Bifurcated = No; Scoring_Function = Energy_Estimate.

The structures of the designed inhibitor−GS complexes were optimized using the program Discover with the cff97 force field[44] and conjugate gradient minimizer. Minimizations were done up to energy change of 0.02 kcal mol^{-1}. The structure of inhibitor−enzyme complex was minimized in two steps. At first positions of hydrogen atoms were optimized and then those of all atoms of active site residues and inhibitor were minimized. Minimized structures were scored using LUDI_1, LUDI_2, and LUDI_3 functions from LUDI program deriving from the Insight package.

Bioassays. The commercially available, fully activated glutamine synthetase from *E. coli* strain W (ATCC 9637, Sigma G3144) was reconstituted with double distilled water at a concentration of 100 nkat mL^{-1}, and stored on ice until used. GS activity was measured by monitoring the release of inorganic phosphate during the physiological full forward reaction, as previously described.[65] Assays were performed at 37 °C for up to 20 min in 50 mM Tris-HCl buffer, pH 7.4 (final volume of 100 μL) in the presence of: 100 mM glutamate, 5 mM ATP, 1 mM NH_4Cl, 25 mM $MgCl_2$, and a limiting amount (30 pkat) of enzyme. Activity was calculated from the initial linear part of reaction rate curves. For each compound, enzyme inhibition was evaluated by adding to the reaction mixture 10 μL of an appropriate dilution of a 10 mM water solution (pH 7.4), so as to obtain the following doses: 1000, 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, and 0.1 μM. At least four measurements were performed for each dose. The concentrations causing 50% inhibition (IC_{50}) of GS activity were estimated utilizing the linear regression equation of enzyme activity values, expressed as percentage of untreated controls, plotted against the logarithm of inhibitor concentration. Confidence limits of IC_{50} values were computed according to the method of Snedecor and Cochran.[66]

For kinetic evaluations, the enzyme was assayed in the presence of increasing concentrations of the inhibitors at varying levels of the substrates. Unvariable substrates were fixed at the same levels as in standard assay. Concentration for the variable substrate ranged from 15 to 80 mM for glutamate, from 0.2 to 1.0 mM for ATP, and from 0.15 to 0.70 mM for ammonia. At least eight doses were evaluated for each substrate, at least in triplicate. In the case of glutamate (competitive inhibition), K_i values were estimated from Lineweaver−Burk plots of activity; at least four inhibitor concentrations, ranging from 0.2- to 2-fold the corresponding IC_{50} value, were tested. In the case of ATP and ammonia (uncompetitive inhibition), K_i values were estimated from Dixon plots of activity by evaluating the effect of at least six inhibitor concentrations, ranging from 0.2- to 1.5-fold the corresponding IC_{50} value, in the presence of at least four substrate levels. Reported values are means ± SEM over results obtained with different inhibitor or substrate concentrations, respectively.

Acknowledgment. This work was supported by KBN grant PBZ-KBN-060/T09/2001. Calculations were done using hardware and software resources, including Accelrys programs, of the Supercomputing and Networking Center in Wrocław. Partial support from the University of Ferrara (within the frame of a bilateral Agreement with the Wrocław University of Technology) is also gratefully acknowledged.

Supporting Information Available: HPLC purity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM050474E