suspended at 37 °C in a 20-mL organ bath with Tyrode's solution aerated with a 95:5 mixture of O_2–CO_2 gas.[20] After equilibration for at least 1 h, cumulative dose–response curves for effects on rate and force of spontaneous contractions were determined by serial additions of the alkaloid in methanol, with at least 10 min between additions.[2] Such experiments were repeated two to six times.

Synaptoneurosomes. Preparation of synaptoneurosomes was as described.[21] Briefly, the cerebral cortex of one guinea pig was homogenized in 7–10 volumes of Krebs–Henseleit buffer (pH 7.4) in a glass–glass homogenizer (5 strokes). The suspension was centrifuged at this point at 1000*g* for 10 min, the supernatant decanted, and the pellet reconstituted in an appropriate volume of buffer to reach a concentration of about 3 mg of protein/mL.

Phosphoinositide Breakdown in Synaptoneurosomes. Phosphoinositide breakdown was measured as described.[22] Briefly, the synaptoneurosome pellet from one guinea pig was resuspended in 10–15 mL of fresh buffer containing 1 μM [^{3}H]inositol (10 μCi/mL). Aliquots of 320 μL of the suspension (~1 mg of protein) were distributed in 5-mL polypropylene tubes and incubated at 37 °C. After 60 min, 20 μL of 200 mM LiCl was added in each tube. Ten minutes later, pumiliotoxin B, analogues, or congeners were added in 20 μL. The final incubation volume was 400 μL. The tubes were gassed briefly with O_2–CO_2, capped, and incubated for 90 min at 37 °C. At the end of the incubation period, the tubes were centrifuged and the tissue was washed with fresh buffer to remove the free [^{3}H]inositol. Then 1 mL of 6% trichloroacetic acid was added, and the tubes were vortexed and centrifuged. Anion-exchange columns (AG 1-X8, formate form) were used to separate the inositol phosphates. The trichloroacetic acid supernatant was added to the column. After washing four times with 3 mL of water to elute free [^{3}H]inositol, [^{3}H]inositol 1-phosphate was eluted with 2 × 1 mL of 200 mM ammonium formate–100 mM formic acid. This eluant was collected in vials. Hydroflour was added (8 mL) and radioactivity determined by liquid scintillation spectroscopy. The trichloroacetic acid precipitate (see above) was resuspended in 0.5 mL of a mixture of aqueous 1 M KCl containing 10 mM inositol and methanol (1:1), and 0.5 mL of chloroform was added. The tubes were mechanically shaken for 5 min, and then centrifuged in order to separate the two phases. Two hundred microliter aliquots from the chloroform phase were placed in individual scintillation vials and evaporated at room temperature. Betafluor was added and radioactivity determined by liquid scintillation spectroscopy to provide an index of [^{3}H]inositol incorporation into [^{3}H]-phosphoinositides. Results are expressed as percent of control response.

Sodium Influx in Synaptoneurosomes. Sodium flux studies were carried out essentially as described by Tamkun and Catterall[23] for synaptosomes. Briefly, aliquots of synaptoneurosome suspensions containing approximately 200–300 μg of protein were preincubated in a volume of 100 μL for 10 min at 37 °C in an incubation buffer containing various test agents. The incubation buffer consisted of 50 mM HEPES (adjusted to pH 7.4 at 36 °C with 50 mM Tris buffer), 130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 1 mg/mL bovine serum albumin (BSA). The ^{22}NaCl, 1.3 μCi/mL, was added in a volume of 150 μL of influx buffer containing 2.66 mM NaCl, 50 mM HEPES-Tris (pH 7.4), 128 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1 mg/mL BSA, and 5 mM ouabain. The final volume was 250 μL. The influx buffer contained test agents in the same concentration as was present in the preincubated samples. Influx of $^{22}Na^+$ was stopped after 10 s by adding 4 mL of cold washing buffer. Samples were immediately collected on a Gelman filter (GN-6, 0.45-μm pore size, Gelman Sciences, Inc., Ann Arbor, MI) and further washed twice with 4 mL of buffer. Washing buffer contained 5 mM HEPES-Tris, 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 1 mg/mL BSA (pH 7.4). Filters were dissolved in Filtron-X (National Diagnostics, Sommerville, NJ) for liquid scintillation counting (efficiency approximately 99%). The uptake of $^{22}Na^+$ through sodium channels was determined by subtracting nonspecific uptake obtained in the presence of 5 μM tetrodotoxin from the total uptake.

The protein content of the synaptoneurosome preparation was determined by precipitation with 6% trichloroacetic acid, centrifugation, and assay of the resulting pellet in 1 M NaOH using the method of Lowry et al.[24]

Phosphoinositide Breakdown in Mouse Atria. Male mice (20–25 g) were decapitated, and hearts were rapidly dissected. Right and left atrial tissue was excised and incubated at 37 °C for 60 min in 1 mL of Krebs–Henseleit buffer (pH 7.4) containing 1 μM [^{3}H]inositol (10 μCi/mL). LiCl was then added (final concentration 10 mM), and after 15 min, agents were added in 50 μL. Incubations were for 30 min at 37 °C. At the end of the incubation, the tissue was blotted, rinsed twice with fresh buffer, and homogenized in 1 mL of 6% trichloroacetic acid. After centrifugation, [^{3}H]inositol phosphates were analyzed in the supernatant by anion-exchange chromatography as described for synaptoneurosomes. Results are expressed as percent of control.

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4-*N*-Hydroxy-L-2,4-diaminobutyric Acid. A Strong Inhibitor of Glutamine Synthetase

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Analogues of glutamic acid were synthesized and evaluated for their inhibitory activity toward glutamine synthetase (EC 6.3.1.2; GS). The title compound, 4-*N*-hydroxy-L-2,4-diaminobutyric acid (NH-DABA), showed a potent inhibitory activity against GS from both sheep brain and soybean. The inhibition is competitive with respect to glutamic acid and the K_i values of sheep brain GS and soybean GS for NH-DABA are 0.007 mmol and 0.021 mmol, respectively. The activity of inhibition is comparable to those of L-methionine sulfoximine and 2-amino-4-(hydroxymethylphosphinyl)butyric acid (phosphinothricin).

Glutamine synthetase (GS; L-glutamate; ammonia ligase; EC 6.3.1.2) is widely distributed in microorganisms, animal tissues, and higher plants, and catalyzes the formation of glutamine from glutamic acid and ammonia.[1] In the

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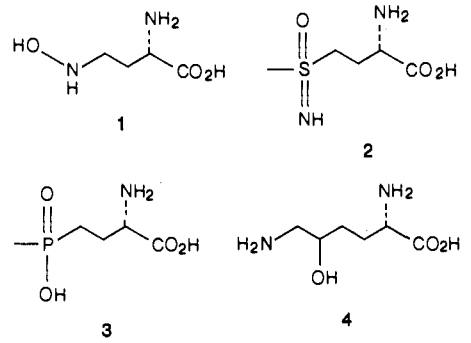
central nervous system, GS not only participates in intermediary metabolism but also detoxifies ammonia and helps regulate glutamic acid and γ-aminobutyric acid (GABA) neurotransmission.[2] Recently, there has been much interest in glutamic acid and glutamine and their connection in tumor cell growth since they have been shown to contribute to cellular growth, including participation in purine and pyrimidine biosynthesis and energy metabolism.[3-7] It has been demonstrated that not only glutamine antagonists such as 6-diazo-5-oxo-L-norleucine (DON) and acivicin but also GS inhibitors such as 5-hydroxylysine (4) and L-methionine sulfoximine (MSO, 2)



have an inhibitory effect on the growth of tumor cells.[8] On the other hand, an accumulation of ammonia caused by the inhibition of GS causes a plant to wither and die. The tripeptidal antibiotic bialaphos[9] shows a herbicidal activity, since one of the strongest inhibitor of GS, phosphinothricin, is contained as the active principle in the molecule.[10] Therefore, the search for new GS inhibitors is important not only in the biological aspect but in the medicinal field.

Known inhibitors[11,12] of GS (1-4) have a common structural feature in that they are α-amino acids and have at least one oxygen atom attached at the 5-position of the amino acid. This indicates that a negative charge on the oxygen atom is essential for the inhibition of GS, and compounds having a phosphorus or a sulfur atom at the 5-position especially exhibit a strong inhibitory activity. Therefore, we synthesized some glutamic acid analogues that satisfied the requirements mentioned above and evaluated their inhibitory activity on GS. Among the compounds tested, 4-*N*-hydroxy-L-2,4-diaminobutyric acid (NH-DABA, 1) has been found to have a potent inhibitory activity against GS. An efficient synthetic route and the strong inhibitory property of NH-DABA on sheep brain and soybean GS are described in this paper.

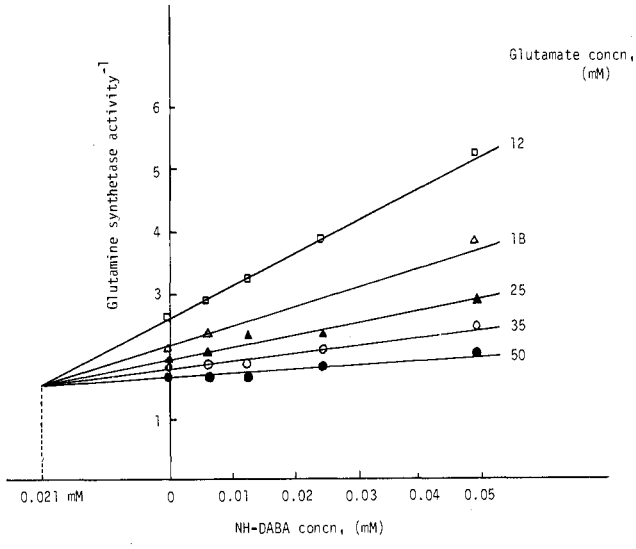


Figure 1. Inhibitory action of 4-*N*-hydroxy-L-2,4-diaminobutyrate (NH-DABA) on soybean glutamine synthetase. Plot of $1/v$ against inhibitor concentration at varying glutamate concentrations.

Table I. Inhibitory Action of Compounds on Sheep Brain GS (Percent Inhibition)[a]

concn, mM	NH-DABA[b]	5-OH-Lys[c]	MSO[d]	AMPB[e]	*N*-Me-HA[f]
0.5	100	22	31	100	8
0.2	92	11	19	96	4
0.1	80	5	9	88	0

[a] Incubation, 37 °C for 15 min. [b] NH-DABA: *N*-hydroxy-L-2,4-diaminobutyrate (1). [c] 5-OH-Lys: 5-hydroxylysine (4). [d] MSO: L-methionine sulfoximine (2). [e] AMPB: L-phosphinothricin (3). [f] *N*-Me-HA: *N*-methylhydroxylamine.

Table II. Inhibitory Action of Compounds on Soybean GS (Percent Inhibition)[a]

concn, mM	NH-DABA[b]	5-OH-Lys[c]	MSO[d]	AMPB[e]	*N*-Me-HA[f]
0.5	84	21	90	100	0
0.2	64	9	76	97	0
0.1	47	5	56	91	0

[a] Incubation, 37 °C for 30 min. [b] NH-DABA: *N*-hydroxy-L-2,4-diaminobutyrate (1). [c] 5-OH-Lys: 5-hydroxylysine (4). [d] MSO: L-methionine sulfoximine (2). [e] AMPB: L-phosphinothricin (3). [f] *N*-Me-HA: *N*-methylhydroxylamine.

Results and Discussion

Chemistry. Reductive coupling of an amine and a carbonyl compound with sodium cyanoborohydride ($NaBH_3CN$) is a useful method for N-alkylation.[13] Since $NaBH_3CN$ also reduces an oxime formed by the reaction of a hydroxylamine and an aldehyde to corresponding alkylhydroxylamine under mild reaction conditions and in good yield, this procedure was adopted in the synthesis of NH-DABA (Scheme I). Thus, reduction of the stereoisomeric mixture of oximes obtained by the reaction of hydroxylamine hydrochloride and L-aspartic β-semialdehyde (5)[14,15] prepared from L-homoserine in three steps, yielded a hydroxylamine derivative, which lactamized to a hydroxamate 7 upon purification. Deprotection of 7 with 30% hydrobromic acid in acetic acid gave 8, which, when hydrolyzed with 5 N hydrochloric acid under reflux, afforded NH-DABA monohydrochloride 1. By this method,

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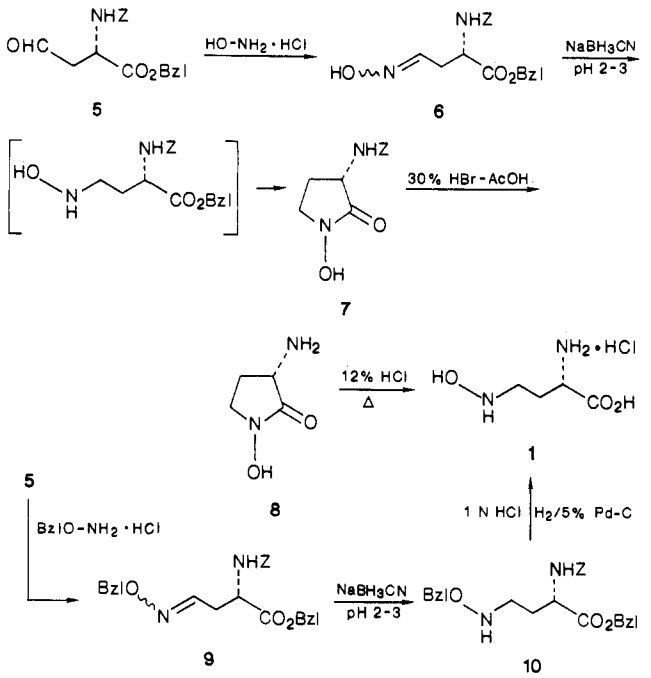
Scheme I



however, NH-DABA was prepared in low yield because of the formation of the lactam **7**. When *O*-benzylhydroxylamine was employed in the place of hydroxylamine, the lactamization was suppressed, and compound **10** was obtained as the sole product. Deprotection of **10** by hydrogenolysis in the presence of hydrochloric acid produced NH-DABA as monohydrochloride in a satisfactory yield.

In Vitro Inhibition Assays. The assay was carried out both on commercially available sheep brain GS and on GS prepared from soybean according to the procedure of Rowe et al.[16,17] GS activity was determined by the γ-glutamylhydroxamate procedure. The inhibitory activity of NH-DABA both on soybean and sheep brain GS was tested at various glutamate and inhibitor concentrations (Figures 1 and 2).

At the concentrations tested in this experiment (0–0.05 mM) NH-DABA is a competitive inhibitor of glutamate with both enzymes. The kinetics of the inhibition of soybean GS by NH-DABA as shown in Figure 1 are very similar to the data obtained for the sheep brain enzyme as shown in Figure 2. From these results, it is possible to calculate K_i values for NH-DABA of 0.021 mM on soybean GS and 0.007 mM on the sheep brain enzyme.

The inhibiting activity of NH-DABA was compared with those of known inhibitors, and the results are summarized in Tables I and II. Thus, under our experimental conditions, NH-DABA inhibits the soybean GS activity by 64% at a 0.2 mM concentration, whereas 2-amino-4-(hydroxymethylphosphinyl)butyric acid (AMPB) inhibits the enzyme by 97% at the same concentration. On the other hand, NH-DABA inhibits the sheep brain GS activity by 92% at a 0.2 mM concentration. At the same concentration, AMPB inhibits the enzyme by 96%.

From the presented results, it is concluded that NH-DABA is a strong inhibitor of sheep brain and soybean GS and is more effective on the sheep brain GS than the soybean GS (Tables I and II).

It is well-known that NH-DABA inhibits GABA-aminotransferase (GABA-T), which requires pyridoxal

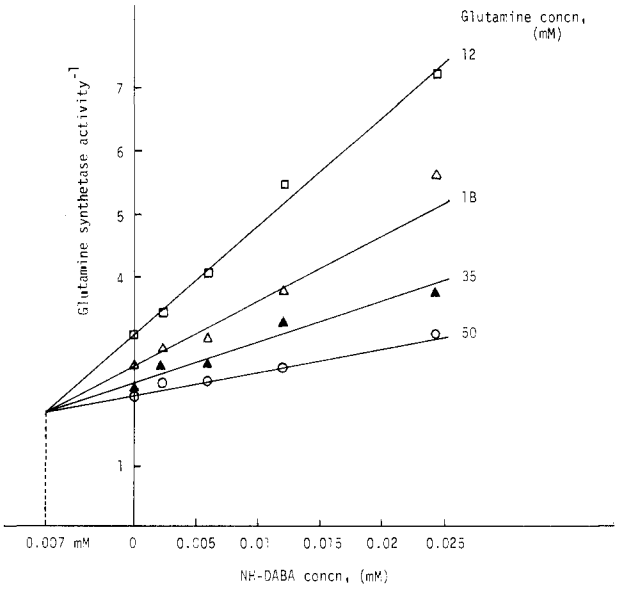


Figure 2. Inhibitory action of 4-*N*-hydroxy-L-2,4-diaminobutyrate (NH-DABA) on sheep brain glutamine synthetase. Plot of $1/v$ against inhibitor concentration at varying glutamate concentrations.

phosphate (PLP) as a cofactor.[18] Since a simple hydroxylamine, *N*-methylhydroxylamine, also inhibits GABA-T, the inhibition is attributable to the formation of a Schiff's base between PLP and the hydroxyamino moiety of the compound.[19] This indicates that the hydroxyamino moiety of NH-DABA probably functions as an amine.

On the other hand, NH-DABA inhibited GS, while *N*-methylhydroxylamine did not inhibit GS (Tables I and II). Since GS does not contain PLP as a cofactor, it is probably the case, therefore, in which the hydroxy group of NH-DABA plays an important role, e.g., functions as a carboxylic acid, and further work is under way to confirm this idea. As mentioned in the beginning, an inhibitor of GS is also expected to have herbicidal or antitumor activities, and the research on these activities of NH-DABA is under investigation.

Experimental Section

GS Activity. GS activity was measured by the formation of γ-glutamylhydroxamate in the synthetase reaction. The reaction mixtures contained enzyme (100 μL), sodium L-glutamate (50 mM), NH_2OH (100 mM), NaATP (10 mM), $MgCl_2$ (20 mM), imidazole–HCl buffer (pH 7.2; 50 mM), 2-mercaptoethanol (25 mM), and inhibitor as indicated, final volume 1.0 mL. After incubation at 37 °C for 15 min, Fe^{3+} was added, and the formation of γ-glutamylhydroxamate was measured at 500 nm (Hitachi-U3200 spectrophotometer) and compared with a control in which no inhibitor was added.

Enzyme Isolation. Soybeans (20 g) soaked in water overnight were extracted in fresh water (80 mL) in a blender and then homogenized in a Teflon homogenizer. The homogenate was centrifuged at 17400*g* for 15 min at 0 °C and brought to 35% saturation with solid $(NH_4)_2SO_4$. The centrifuged supernatant solution was subjected to 65% saturation with solid $(NH_4)_2SO_4$ and centrifuged (17400*g*, 15 min, 0 °C). The centrifuged precipitate was suspended in water (9 mL), imidazole–HCl buffer (pH 7.2, 1 mL), and 2-mercaptoethanol (1 M, 0.2 mL) and used for the assay.

Analytical Procedures. Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. Elemental analyses were performed by K. Koike, Pharmaceutical

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Institute of Tohoku University, and were in agreement within ±0.4% with the proposal structures. Optical rotations were measured with a JASCO DIP-340 polarimeter. Mass spectra were recorded with a Hitachi M-52G (EI) and a JEOL JMS-01 SG-2 spectrometer (FD). IR spectra were recorded on a JASCO infrared spectrometer (Model A-100S). ^{1}H NMR spectra were recorded on JEOL spectrometers (Model PMX 60 SI, 60 MHz, and FX 100, 100 MHz). The ^{1}H NMR spectra were recorded with Me_4Si as internal standard. IR and NMR spectral data were recorded for all numbered compounds and were judged to be consistent with the assigned structures.

L-Aspartic β-Semialdehyde (5). To a solution of L-homoserine (Tokyokasei Co.; 5.95 g, 50 mmol) and triethylamine (10.5 mL, 75 mmol) in water (30 mL) was added benzyl *S*-(4,6-dimethylpyrimid-2-yl)thiolcarbonate (15.1 g, 55 mmol) in dioxane. After being stirred at room temperature for 16 h, the reaction mixture was extracted with ethyl acetate (20 mL × 2). The aqueous layer was acidified with 5 N HCl and extracted with ethyl acetate (30 mL × 3). The combined organic layer was washed with 1 N HCl and brine and dried over Na_2SO_4. After filtration, the extract was concentrated to give crude carbobenzoxy-L-homoserine (11). To a stirred solution of 11 in dimethylformamide (150 mL) and CH_2Cl_2 (100 mL) were added dicyclohexylamine (9.95 mL, 50 mmol) and benzyl bromide (6.0 mL, 50 mmol). After the mixture was stirred at room temperature for 24 h, the precipitate was filtered off, and the filtrate was concentrated to dryness. The residue was dissolved in ethyl acetate, washed with 1 N HCl, water, 5% $NaHCO_3$, and water, successively, and dried over Na_2SO_4. The extract was concentrated, and the residue was chromatographed on a silica gel column with hexane/ethyl acetate (1/1) to give benzyl ester 12 as colorless oil (13.4 g, 78% from L-homoserine). To a stirred suspension of pyridinium chlorochromate (Aldrich Chemical Co.; 6.20 g, 28.6 mmol) and Celite (5 g) in CH_2Cl_2 (30 mL) was added benzyl ester 12 (4.9 g, 14.3 mmol) in CH_2Cl_2 (30 mL) in one portion, and the mixture was stirred at room temperature for 3 h. The reaction mixture was subjected to a Florisil column eluting with ether. After the solvent was removed, the remaining oily residue was chromatographed on a silica gel column eluting with hexane/ethyl acetate (2/1) to afford aspartic β-semialdehyde 5 as colorless needles (3.43 g, 70%): mp 80.5–82 °C; $[\alpha]^{20}_D$ +20.0° (*c* 0.65, $CHCl_3$); ^{1}H NMR (60 MHz, $CDCl_3$) δ 3.00 (2 H, d, *J* = 6 Hz), 4.4–4.8 (1 H, m), 5.03 (2 H, s), 5.09 (2 H, s), 5.66 (1 H, br d, *J* = 10 Hz), 7.23 (10 H, s), 9.57 (1 H, s).

Preparation of 4-*N*-Hydroxy-L-2,4-diaminobutyric Acid (NH-DABA, 1). Method A. A mixture of aldehyde 5 (2.74 g, 8.0 mmol) and NH_2OH·HCl (840 mg, 14 mmol) in pyridine (10 mL) and ethanol (15 mg) was stirred at room temperature for 3 h. The reaction mixture was concentrated, and the oily residue was chromatographed on a silica gel column eluting with hexane/ethyl acetate (1/1) to give a geometrically isomeric mixture of oximes 6 as a colorless oil (2.67 g, 94%). To a solution of a mixture of oximes 6 (1.81 g, 5.08 mmol) in methanol (60 mL) was added $NaBH_3CN$ (Aldrich; 321 mg, 5.1 mmol), and the reaction mixture was stirred at room temperature for 1.5 h. The pH of the solution was kept at 2–3 by addition of methanolic HCl solution during the period of the reaction. The reaction mixture was neutralized with 1 N NaOH and concentrated by evaporation. The residue was subjected to silica gel column chromatography eluting with $CHCl_3$/methanol (4/1) to afford a pyrrolidone 7 as a white powder (369 mg, 29%): mp 173–174 °C; FD-MS, *m/z* 250 (M^+); ^{1}H NMR (60 MHz, CD_3OD) δ 1.3–2.3 (2 H, m), 3.10 (2 H, br dd, *J* = 4.5 and 8.6 Hz), 3.80 (1 H, br t, *J* = 9.0 Hz), 4.66 (2 H, s), 6.86 (5 H, s). To a mixture of 30% HBr in acetic acid (1.2 mL) and trifluoroacetic acid (0.5 mL) was added pyrrolidone 7 (312 mg, 1.25 mmol), and the mixture was stirred at room temperature for 70 min. Ether was added to the reaction, and the resulting precipitate was filtered off. The precipitate was dissolved in water and passed through a column filled with Dowex 50W × 4 (H^+), eluting with 1 N NH_3 solution. The eluent was concentrated in vacuo to give the amine 8 as colorless plates (142 mg, 98%): mp 155–156 °C dec; FD-MS, *m/z* 117 (M + H)$^+$; ^{1}H NMR (100 MHz, D_2O) δ 1.8–2.3 (1 H, m), 2.4–2.8 (1 H, m), 3.74 (2 H, dd, *J* = 5 and 8 Hz), 4.07 (1 H, t, *J* = 8.5 Hz). A solution of 8 (101 mg, 0.87 mmol) in HCl (12%, 5 mL) was boiled with reflux for 4 h. The reaction mixture was concentrated, the remaining oily residue was mixed with water (2 mL), and the resultant solution was concentrated in vacuo. This operation was repeated three times to ensure removal of excess HCl to give NH-DABA hydrochloride 1, which recrystallized from water/methanol/ether (1:2:2) to give a white powder (105 mg, 71%): mp 182–183 °C dec; $[\alpha]^{20}_D$ +5.1° (*c* 0.19, H_2O); FD-MS, *m/z* 135 (M + H)$^+$; ^{1}H NMR (100 MHz, D_2O) δ 2.3–2.7 (2 H, m), 3.69 (2 H, br t, *J* = 7.5 Hz), 4.19 (1 H, t, *J* = 7 Hz). Anal. ($C_4H_{11}N_2O_3Cl$) C, H, N.

Method B. A mixture of aldehyde 5 (341 mg, 1 mmol) and *O*-benzylhydroxylamine hydrochloride (160 mg, 1 mmol) in pyridine (2 mL) and ethanol (2 mL) was stirred at room temperature for 6 h. After the solvent was removed by evaporation, the residue was chromatographed on a silica gel column eluting with hexane/ethyl acetate (5/1) to give a geometrically isomeric mixture of benzyloxyimines 9 as a white powder (420 mg, 94%). To a solution of the mixture of benzyloxyimines 9 (400 mg, 0.9 mmol) in methanol (5 mL) was added $NaBH_3CN$ (90 mg, 1.45 mmol), and the reaction mixture was stirred at room temperature for 2 h. The pH of the solution was kept at 2–3. The reaction mixture was neutralized with 1 N NaOH and concentrated. Water was added to the oily residue, which was then extracted with ethyl acetate. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column eluting with hexane/ethyl acetate (4/1) to yield benzylhydroxyamine 10 (287 mg, 71%) as a white powder: mp 64–65 °C; MS (*m/z*) 448 (M^+); ^{1}H NMR (60 MHz, $CDCl_3$) δ 1.6–2.2 (2 H, m), 2.92 (2 H, br t, *J* = 6 Hz), 4.2–4.7 (1 H, m), 4.62 (2 H, s), 5.07 (2 H, s), 5.12 (2 H, s), 5.49 (1 H, br s), 5.77 (1 H, br d, *J* = 8 Hz), 7.22 (5 H, s), 7.27 (10 H, s). To a solution of 10 (245 mg, 0.55 mmol) in methanol (5 mL) were added 1 N HCl (1.65 mL) and 5% Pd–C (50 mg), and the mixture was stirred at room temperature for 3 h under hydrogen atmosphere. The catalyst was filtered off, and the filtrate was concentrated. The remaining oily residue was mixed with water, and the resultant solution was concentrated to give NH-DABA hydrochloride 1 (56 mg, 60%).

Acknowledgment. This research was supported in part by Grant-in-Aid for Special Project Research (No. 60119003) from the Ministry of Education, Science and Culture. We are grateful to Pharmaceutical Research Laboratories, Meiji Seika Co., Ltd. for giving us the sample of phosphinothricin.

Registry No. 1·HCl, 111821-57-9; 1 (free base), 111821-59-1; 2, 15985-39-4; 3, 35597-44-5; 4, 1190-94-9; 5, 58578-45-3; (*Z*)-6, 111772-92-0; (*E*)-6, 111772-96-4; 7, 111772-93-1; 8, 111821-58-0; (*Z*)-9, 111772-94-2; (*E*)-9, 111772-97-5; 10, 111772-95-3; 11, 35677-88-4; 12, 58578-44-2; H-Hse-OH, 672-15-1; $PhCH_2ONH_2$·HCl, 2687-43-6; $PhCH_2Br$, 100-39-0; glutamine synthetase, 9023-70-5.