Articles

Drug Latentiation by γ-Glutamyl Transpeptidase

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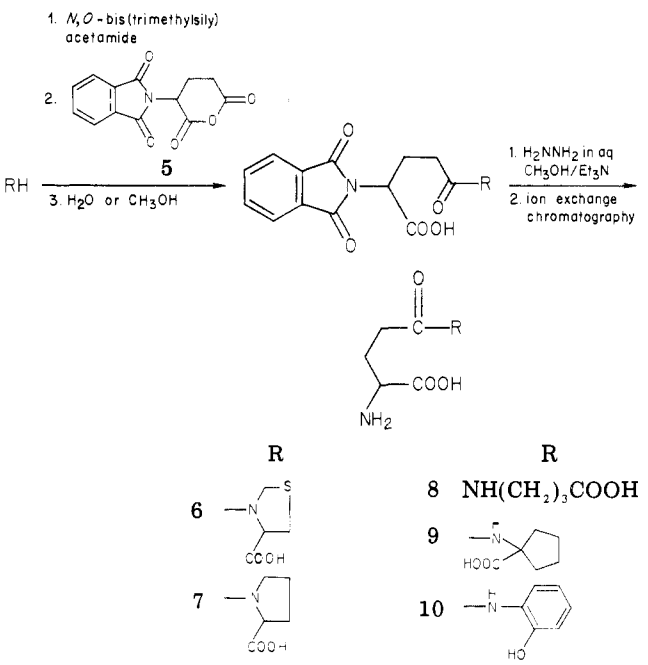
The *N*-γ-glutamyl derivatives of L-thiazolidine-4-carboxylic acid, 4-aminobutyric acid, 1-aminocyclopentanecarboxylic acid, 2-aminophenol, and *p*-fluoro-L-phenylalanine (compounds **6**, **8**, **9**, **10**, and **12**, respectively) were synthesized using the synthon phthaloylglutamic anhydride. Their relative rates of cleavage by the enzyme γ-glutamyl transpeptidase (γ-GT) were determined in order to evaluate the possibility for their selective release by this enzyme which is elevated in certain pathological conditions. Compounds **6**, **8**, and **9** were not readily solvolyzed by γ-GT, but compounds **10** and **12**, as well as the N-γ-glutamylated derivatives of 3- and 4-aminophenol, were readily cleaved.

The chemical modification of a biologically active substance to give a new compound from which the active substance can be liberated in vivo by enzymatic action has been defined by Harper[1] as drug latentiation. Such latentiated drug molecules constitute a special category of prodrugs.[2]

The recent reports that the enzyme γ-glutamyl transpeptidase (γ-GT) is found in high concentrations in certain experimental tumors[3] and in the serum of chronic alcoholics with suspected alcohol-induced liver injury[4]—in addition to the levels normally found in the brush border of the proximal convoluted tubules of the kidneys and in the choroid plexus of the brain[5]—suggest that this enzyme might be exploited for site-selective delivery of drugs.[6] This can be accomplished by chemically modifying drugs that contain an amino functional group in the molecule as γ-glutamyl amide derivatives of the amino acid glutamic acid. This concept, first proposed by Szewczuk et al.,[7] has been applied by Kyncl and others[8] to dopamine by converting the latter to its γ-glutamyl derivatives, thereby producing kidney-specific dopamine precursors with selective renal vasodilator activities. In addition, γ-glutamyl-Dopa[9] and a series of N-acetylated γ-glutamyl derivatives of sulfamethoxazole[10] have been designed as kidney-specific prodrugs. The transport inhibitory amino acid

Scheme I



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(6) A referee has pointed out that the action of γ-GT may not always be involved, because certain γ-glutamyl compounds are transported as such; in these instances, the liberation of the moiety bound to the γ-glutamyl residue would take place intracellularly, very likely mediated by the intracellular enzyme γ-glutamylcyclotransferase.

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adamantanine, however, could not be liberated in vivo as its γ-glutamyl derivative due to the latter's inactivity as a substrate for γ-GT.[11]

We describe here the preparation of some γ-glutamyl derivatives of a selected series of amino-functionalized substances of varying structures and of current interest to us that might benefit from latentiation. These γ-glutamyl compounds were subjected to enzymatic glutamyl cleavage in vitro by γ-GT in order to assess the general applicability of this concept to these compounds and to ascertain the structural features necessary for optimal hydrolysis with this enzyme.

The compounds selected for latentiation are L-thiazolidine-4-carboxylic acid (**1**), 4-aminobutyric acid (GABA, **2**), 1-aminocyclopentanecarboxylic acid (cycloleucine, **3**), *p*-fluoro-L-phenylalanine (**4**), and the 4-, 3-, and 2-aminophenols. Compound **1**, as the free amino acid or as its arginine salt, is being used in Europe for the treatment of alcoholic hepathopathies.[12] Its CNS toxicity[13] suggested to us that a latentiated form providing slow release of **2** might be less toxic and also more selective in alcoholics with markedly increased hepatic γ-GT. Low brain levels of **2** prevail in alcoholic withdrawal seizures[14a] and in Huntington's disease.[14b] γ-Glutamyl-GABA could conveivably serve as a GABA delivery prodrug, since localization of γ-GT in the choroid plexus suggests that this enzyme may mediate the transport of γ-glutamylated derivatives into the CSF whose access is normally blocked by the blood–brain barrier.[14b,c,6] The *N*-γ-glutamyl derivative of 4-aminophenol, a naturally occurring substance isolated from the mushroom species *Agaricus biosporus*,[15] as well as the synthetically prepared isomer, *N*-γ-glutamyl-3-aminophenol, show antitumor activities against B-16 melanoma.[16] Cycloleucine (**3**) and *p*-fluorophenylalanine also exhibit antitumor activities of varying degrees.[17,18]

Chemistry. The general procedure for the synthesis of the γ-glutamyl derivatives of **1**, L-proline, **2**, **3**, and 2-aminophenol is depicted in Scheme I. The synthon *N*-phthaloyl-L-glutamic anhydride (**5**) was selected, since nucleophilic attack occurs regioselectively at the γ-carbonyl group of **5**[19] giving rise to amide bond formation at this γ position. The amino acids, as well as the *o*-aminophenol to be γ-glutamylated, were first protected as their respective trimethylsilyl derivatives in an inert solvent before **5** was added for coupling. It was not feasible to characterize the intermediate *N*-phthaloyl-L-glutamyl derivatives as they were isolable only as glassy foams, but after hydrazinolysis of the phthaloyl group, the deprotected γ-glutamyl compounds were purified by ion-exchange chromatography and isolated in pure form.

Scheme II

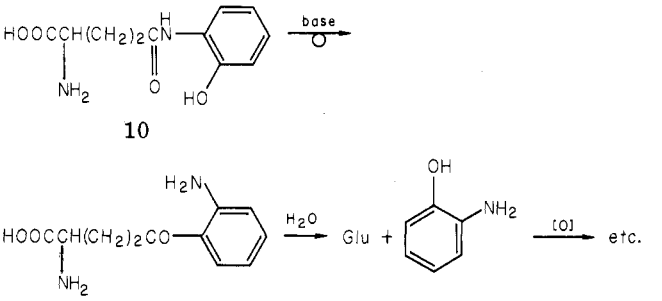

Table I. Solvolysis of γ-Glutamyl Compounds Catalyzed by γ-Glutamyl Transpeptidase

compound	rel act.[a]
S-Methylglutathione	100[b]
N-γ-L-glutamyl-L-thiazolidine-4-carboxylic acid (**6**)	5
N-γ-L-glutamyl-L-proline (**7**)	4
N-γ-L-glutamyl-4-aminobutyric acid (**8**)	9
N-γ-L-glutamyl-L-2-aminobutyric acid	95
N-γ-L-glutamyl-1-aminocyclopentanecarboxylic acid (**9**)	19
N-γ-L-glutamyl-glycine	70
N-γ-L-glutamyl-*p*-fluoro-L-phenylalanine (**12**)	64
N-γ-L-glutamyl-L-phenylalanine	67
N-γ-L-glutamyl-2-aminophenol (**10**)	∼95[c]
N-γ-L-glutamyl-4-aminophenol	98[d]
N-γ-L-glutamyl-3-aminophenol	96[d]

[a] Each value represents the average of two sets of duplicate or triplicate determinations from two separate experiments. [b] Specific activity = 32.5 nmol of γ-glutamyl hydroxymate formed min^{-1} (mg of protein)$^{-1}$. This value represents the average of five separate determinations, each done in triplicate. [c] Estimated; see text for explanation. [d] Duplicate determinations only.

Generally, an anion-exchange resin in the acetate form was used for this chromatographic purification step; however, for **10**, this procedure led to the formation of colored decomposition products. Use of a weakly acidic (carboxylic acid) cation-exchange resin gave the desired **10**, albeit in low yield. The low yield can be accounted for in part by a base-catalyzed N → O acyl migration of the glutamyl group after hydrazinolysis, followed by solvolysis to *o*-aminophenol, thereby giving rise to colored products upon air-oxidation of the latter—as shown in Scheme II.

Racemic *p*-fluorophenylalanine was converted to its *N*-trifluoroacetyl derivative, and the L isomer was isolated following enzymatic hydrolysis of the trifluoroacetyl group with carboxypeptidase A,[20] the trifluoroacetyl D isomer remaining unhydrolyzed. Since the trimethylsilyl derivative of *p*-fluoro-L-phenylalanine (**4**) was not easily prepared due to the latter's insolubility, the peptide-forming step (Scheme I) was modified by coupling the free amino acid with **5** in glacial acetic acid.[21] Under these conditions, the intermediate is likely the mixed anhydride **11**. Subsequent dephthaloylation and workup followed Scheme I to give **12**.

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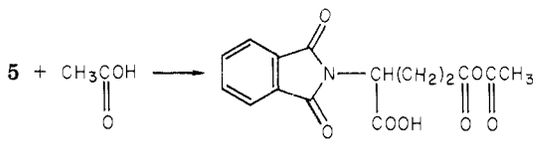

11

All of the γ-glutamyl derivatives synthesized above gave on chemical-ionization mass spectrometry (CIMS) a fragment ion at m/e 130 characteristic for the γ-glutamyl group and fragment ions corresponding to the protonated molecular ion of the C-terminal group,[22] thereby adding credence to the assigned structures. Absence of trace quantities of α isomers was assured by the ion-exchange chromatographic purification procedure used, and this was confirmed by CIMS.[22]

Biological Results. Since the compounds listed in Table I represent γ-glutamyl derivatives of diverse structures, it was necessary to use an assay system uniformly applicable to all of them. By utilizing hydroxylamine as the acceptor for the γ-glutamyl group and determining the formation of γ-glutamyl hydroxamate,[23] we could assess the degree of solvolysis of the γ-glutamyl linkage catalyzed by γ-GT. While this system may not necessarily accurately reflect the *transpeptidase* reaction per se, it has the advantage of permitting the comparison of the compounds under study using the same assay procedure, and does in fact measure the enzymatic cleavage of the γ-glutamyl group from the drug moiety in its latentiated form, thereby fulfilling our objectives.

The γ-glutamyl hydroxamate is complexed with ferric ions and the colored complex measured spectrophotometrically. Since the color yield for this reaction is low for substrates less active than glutathione, it was necessary to enhance the transpeptidase reaction by addition of maleate to the system. Maleate has been shown to increase the formation of γ-glutamyl hydroxamate about 4- to 5-fold.[24] Using a commercially available γ-GT preparation, glutathione as substrate and hydroxylamine as acceptor, we found that maleate addition indeed stimulated the reaction nearly 4-fold (data not shown). The assay system was therefore standardized to include maleate.

The relative activities of the various γ-glutamyl derivatives with γ-GT are tabulated in Table I. *S*-Methylglutathione[24] was used as a standard glutamyl donor, whose activity was set at 100. Because of the possible nonuniformity of the maleate effect in this assay, each test compound (except for the γ-glutamylaminophenols) was compared against a known γ-glutamyl derivative of its own structural type; e.g., **6** was compared against γ-glutamyl-L-proline (**7**), **8** against *N*-γ-L-glutamyl-L-2-aminobutyric acid, and **12** against *N*-γ-L-glutamyl-L-phenylalanine. The activity of **9** was compared against γ-glutamylglycine since no active, naturally occurring γ-glutamyl analogue for cycloleucine—an α,α-disubstitued glycine—was available. It is known that *N*-γ-glutamyl-L-α-aminoisobutyric acid[25] and *N*-γ-glutamyladamantanine[11] are not substrates for γ-GT.

The results (Table I) were predictably variable. The γ-glutamyl derivatives of thiazolidine-4-carboxylic acid (**6**), 4-aminobutyric acid (**8**), and cycloleucine (**9**) were not readily cleaved by γ-GT. While this limited data do not permit generalizaitons, the above represent the γ-glutamyl derivatives of (a) an α-imino acid, (b) a γ-amino acid, and (c) an α,α-disubstituted glycine. The lack of basicity of the imino nitrogen of **1** ($pK_a = 6.2$[26]), apparently did not influence the ease of hydrolysis of **6**, since its activity was similar to the proline analogue **7** (pK_a of L-proline = 10.6[27]), which was a poor substrate as also previously noted.[28]

N-γ-Glutamyl-*p*-fluoro-L-phenylalanine (**12**) and the *N*-γ-glutamyl aminophenols were readily solvolyzed by the enzyme. The relative activity for the 2-aminophenol derivative (**10**) was estimated and could not be accurately assessed because the product of the reaction, viz., *o*-aminophenol, also complexed with the $FeCl_3$ reagent to produce an orange-brown chromophore secondary to the rapid initial reaction with the γ-glutamyl hydroxamate formed, and this interfered with the colorimetric determination of the latter. Since no color was produced by blanks which contained all the components of the assay except for the enzyme and since *o*-aminophenol itself when treated with the $FeCl_3$ reagent also gave the same orange-brown color, it is clear that *o*-aminophenol was generated only by the action of γ-GT on **10**. Neither the products from *N*-γ-L-glutamyl-4-aminophenol nor *N*-γ-L-glutamyl-3-aminophenol gave rise to the secondary color reaction observed with **10**.

These results extend the previous work of others[8,9] and suggest that it is possible to latentiate drugs containing an amino functional group in the molecule as their *N*-γ-glutamyl derivatives and subsequently to liberate the parent drug by the action of γ-GT. While not universally applicable to all structural types (e.g., γ-glutamyl derivatives of secondary amines and of primary amines where the amino groups are on tertiary carbon appear to be poorly solvolyzed by γ-GT, possibly due to steric hindrance), drug latentiation within these limitations appears to be worthy of further exploration.

Experimental Section

Melting points were determined on a Fisher-Johns melting apparatus and are corrected to reference standards. Optical rotations were measured in a Perkin-Elmer Model 141 polarimeter. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. The following spectrophotometers were used: IR, Beckman IR-10; UV–visible, Beckman Acta MVI; the CIMS of the target compounds have already been reported.[22]

γ-Glutamyl transpeptidase, *S*-methylglutathione, γ-glutamyl hydroxamate, and *p*-fluoro-DL-phenylalanine were purchased from Sigma Chemical Co., St. Louis, MO. *N*-γ-L-Glutamyl-L-phenylalanine was purchased from Aldrich Chemical Co., Milwaukee, WI. The *N*-γ-glutamyl derivatives of 4- and 3-aminophenols were kindly supplied by Dr. Andre Rosowsky, Sidney Farber Cancer Institute, Boston, MA.

Unless otherwise noted, silica gel GF plates (Analtech, Inc., Newark, DE) were used for thin-layer chromatography (TLC). The following solvent systems were used: solvent A, butanol–acetic acid–H_2O (4:1:1); solvent B, $CHCl_3$–AcOH (4:1). Analytical grade, ion-exchange resins (Bio-Rad Laboratories, Richmond, CA) were used for ion-exchange chromatography. A micro fraction collector (Model FC-80H; Gilson Medical Electronics, Inc., Middleton WI) was used to collect 1- to 2-mL fractions from the column, and effluents were analyzed by spotting on TLC plates (silica gel), spraying the spots with an ethanolic ninhydrin spray reagent, and heating in a 120 °C oven for 3 to 5 min. Fractions giving purple colors were combined and lyophilized.

***N*-Phthaloyl-L-glutamic Anhydride (5).** This synthon was prepared[29] as needed. Since the optical rotations of the product

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differed slightly from batch to batch, the optical rotations of the sample used are recorded in the individual synthetic procedures that follow.

General Procedure for the Synthesis of γ-Glutamyl Compounds (Scheme I). *N*-γ-L-Glutamyl-L-thiazolidine-4-carboxylic Acid (6). To a suspension of acetonitrile was added 1.9 mL (97.7 mmol) of *N,O*-bis(trimethylsilyl)acetamide, and the mixture was heated under reflux for 1 h. The resulting solution was cooled to room temperature, *N*-phthaloyl-L-glutamic anhydride [**5**; 1.01 g, 3.9 mmol; $[\alpha]^{25}_D$ −43.4° (*c* 1.8, dioxane)] was added, and stirring was continued overnight at room temperature. The slightly cloudy solution was filtered, and the filtrate was evaporated in vacuo to near dryness without heating. The oily residue was taken up in 9.8 mL of 1 M Et_3N in MeOH, and 2.9 mL of 2 M hydrazine in 50% aqueous MeOH was added. The reaction mixture was allowed to stand at room temperature for 3 days, during which time a precipitate formed. This precipitate, which was chromatographically identical with phthalic acid hydrazide on TLC (solvent A), was removed by filtration, and the filtrate was evaporated in vacuo to near dryness. The semisolid product was suspended in 20 mL of H_2O, and the pH was adjusted to 4 by the addition of 1 N HCl. After standing for 3 h at room temperature, the mixture was filtered to remove suspended solids, and the filtrate was lyophilized. The lyophilized material was taken up in 10 mL of H_2O, the solution was filtered, and the filtrate was charged on a 1.5 × 23 cm column of AG 1-X8 (acetate form, 200–400 mesh) anion-exchange resin. The column was washed with H_2O followed by 0.5 N AcOH, and the product was then eluted with 1 N AcOH. The two fractions that gave pink ninhydrin reactions were separately lyophilized. The first fraction (15 mg) was glutamic acid (by IR). The second fraction (650 mg) was recrystallized from water, and the product was collected, washed with MeOH, and dried in vacuo to give 410 mg (40% overall yield) of **6**: mp 205–207 °C dec; $[\alpha]_D$ −93.0° (*c* 1.00, H_2O); IR (Nujol) 1710 (sh, acid CO), 1680 (amide CO), 1635 (br d, COO^-, NH_3^+) cm^{-1}. Anal. ($C_9H_{14}NO_5S$) C, H, N.

***N*-γ-L-Glutamyl-L-proline (7).** This compound was prepared essentially according to the general procedure above using 460 mg (4.0 mmol) of L-proline. Final yield of **7** that had been thoroughly washed with H_2O and MeOH was 330 mg (34%): mp 187–189 °C; $[\alpha]_D$ −75.7° (*c* 1.0, H_2O) [lit.[30] mp 194–195 °C; $[\alpha]_D$ −73.4° (*c* 0.5, H_2O)]; IR (KBr) 1725 (acid CO), 1680 (br d, amide CO), 1625 (br d, COO^- and NH_3^+) cm^{-1}. Anal. ($C_{10}H_{16}N_2O_5$) C, H, N.

***N*-γ-L-Glutamyl-4-aminobutyric Acid (8).** From 206 mg (2.0 mmol) of 4-aminobutyric acid was obtained 160 mg (35% yield) of **8**: mp 177–180 °C; $[\alpha]^{26}_D$ +7.01° (*c* 0.99, H_2O) [lit.[31] mp 177–179 °C; $[\alpha]^{17}_D$ +7.3° (*c* 0.9, H_2O)]; IR (KBr) 3300 (NH amide), 1690 (acid CO), 1640 (amide CO), 1580 (amino acid 2). Anal. ($C_9H_{16}N_2O_5$) C, H, N.

***N*-γ-L-Glutamyl-1-aminocyclopentanecarboxylic Acid (γ-Glutamylcycloleucine) (9).** From 260 mg (2.0 mmol) of 1-aminocyclopentanecarboxylic acid (cycloleucine) was obtained, after recrystallization from MeOH, a total of 205 mg (40% yield) of **9**: mp 203–205 °C; $[\alpha]^{27}_D$ −30.4° (*c* 1.0, H_2O); IR (Nujol) 3350 (amide NH), 1710 (acid CO), 1660 (amide CO), 1610 (br d, COO^-, NH_3^+), 1530 (amide II) cm^{-1}. Anal. ($C_{11}H_{18}N_2O_5$) C, H, N.

***p*-Fluoro-L-phenylalanine (4) (Composite Procedure[20]).** *N*-(Trifluoroacetyl)-*p*-fluoro-DL-phenylalanine[32] (7.0 g, 0.025 mol) was suspended in 200 mL of H_2O and stirred vigorously while the pH was adjusted to 7.2 with 2 N NaOH with an automatic titrator. The resulting clear solution was placed in a water bath at 37 °C, 10 mg of DFP-treated carboxypeptidase A type II (Sigma Chemical Co., St. Louis, MO) was added, and the pH was maintained at 7.2 using an automatic pH maintenance apparatus. The solution was stirred gently overnight at 37 °C. The solution was then adjusted to pH 5 with 2 N HCl, decolorized with charcoal, and filtered. The filtrate was adjusted to pH 3 with 1 N HCl, and the acidified solution was extracted 5 times with a total of 500 mL of EtOAc to remove *N*-(trifluoroacetyl)-*p*-fluoro-D-phenylalanine. The aqueous layer was concentrated to 100 mL by boiling, and the concentrate was decolorized again with charcoal. The filtrate was concentrated to approximately 40 mL, and upon cooling, crystallization occurred. The crystals were collected, and the procedure of boiling, decolorizing, and crystallizing was repeated two times to give a total of 1.69 g (74% yield) of white needles. Recrystallization again from H_2O and drying over P_2O_5 at 57 °C gave 1.46 g (64% yield) of the titled compound: mp 210–225 °C dec; $[\alpha]^{26}_D$ −22° (*c* 0.94, H_2O) [lit.[20b] mp of a hemihydrate 250–255 °C dec; $[\alpha]^{26}_D$ −23° (*c* 2, H_2O)]. Anal. ($C_9H_{10}NO_2F$) C, H, N, F.

***N*-γ-L-Glutamyl-*p*-fluoro-L-phenylalanine (12).** *p*-Fluoro-L-phenylalanine (**4**; 350 mg, 1.9 mmol) and **5** (520 mg, 2 mmol), $[\alpha]^{24}_D$ −43.1° (*c* 2.0, dioxane), were coupled in 3 mL of glacial acetic acid at 60–70 °C for 30 min. The clear solution was lyophilized, and the residual product was dissolved in a mixture of 5 mL of 1 M Et_3N in MeOH and 1.5 mL of 2 M hydrazine in 50% aqueous MeOH. Hydrazinolysis was allowed to proceed at room temperatue for 24 h, after which time the reaction mixture was worked up as described in the general procedure. The middle fraction from ion-exchange chromatography weighing 370 mg was dissolved in H_2O, absolute EtOH was added, and the solution evaporated in vacuo. The resulting crystals were washed with MeOH and absolute EtOH to give 200 mg (34% yield) of **12**: mp 195–197 °C; IR (KBr) 3400 (amide NH), 1720 (acid CO), 1670–1620 (br d, amide CO), 1510 (amide II) cm^{-1}. Anal. ($C_{14}H_{17}N_2O_5F$) C, H, N, F.

***N*-γ-Glutamyl-2-aminophenol (10).** The coupling of 220 mg (2.0 mmol) of *o*-aminophenol and 520 mg (2.0 mmol) of **5**, $[\alpha]^{25}_D$ −45.5° (*c* 1.8, dioxane), followed the general procedure. Hydrazinolysis produced orange-brown solids; however, the desired product also appeared to be present (by TLC in solvent A). The water-soluble material was purified by ion-exchange chromatography on Bio-Rex 70 cation-exchange resin (H^+ form, 100–200 mesh) eluting with water. Workup gave 40 mg (8% yield) of **10**: mp 193–195 °C; IR (KBr) 3380 (amide NH), 3060 (br d, phenolic OH), 1655 (amide CO), 1590 (br d, NH_3^+ and COO^-), 1540 (amide II), 735 (aromatic ortho substitution). Anal. ($C_{11}H_{14}N_2O_4$) C, H, N.

Solvolysis of γ-Glutamyl Compounds Catalyzed by γ-GT. We assayed the compounds listed in Table I for activity with γ-GT using a commercial preparation of γ-GT (lot no. 29C-9540) which, according to the supplier, was prepared by the procedure of Orlowski and Meister.[23] The enzyme assay described in the following section is a modification of the procedure described by Tate and Meister.[24]

General Assay Conditions. The final volume (1 mL) contained Tris-HCl buffer (0.12 M; pH 8.0), γ-glutamyl compound (10 mM), hydroxylamine hydrochloride (200 mM), sodium maleate (50 mM), $MgCl_2$ (0.7 mM), and γ-GT (6 units). The stock solutions of the enzyme and the γ-glutamyl compounds were prepared fresh just prior to the assay. After incubation at 37 °C in a water bath for 20 min, ferric chloride reagent[33] (1.5 mL) was added. The solutions were allowed to stand in an ice bath for 30 min, and the reaction mixture was subjected to centrifugation and/or membrane (0.45 μm) filtration to remove the denatured protein. The formation of γ-glutamyl hydroxamate was determined spectrophotometrically at 535 nm, blanks for each compound which lacked only the enzyme being subtracted. A standard curve prepared from 0 to 200 μmol of γ-glutamyl hydroxamate, worked up in identical manner as above, gave a linear plot of absorbance vs. concentration.

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