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Mapping of the Active Site of Rat Kidney γ-glutamyl Transpeptidase Using Activated Esters and Their Amide Derivatives

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Abstract—The enzyme γ-glutamyl transpeptidase (GGT), implicated in many physiological processes, catalyses the transfer of a γ-glutamyl from a donor substrate to an acyl acceptor substrate, usually an amino acid or a peptide. In order to investigate which moieties of the donor substrate are necessary for recognition by GGT, the structure of the well-recognized substrate L-γ-glutamyl-*p*-nitroanilide was modified. Several activated esters and their amide derivatives were synthesized and used as substrates. Kinetic (K_m and V_{max}) and inhibition constants (K_i) were measured and reveal that almost the entire γ-glutamyl moiety is necessary for recognition in the binding site of the donor substrate. The implied presence of certain complementary amino acids in this substrate binding site will allow the more rational design of various substrate analogues and inhibitors.

Introduction

γ-Glutamyl transpeptidase (GGT; EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme implicated in leukotriene biosynthesis[1] and cellular detoxification through the formation of mercapturic acids.[2] GGT is also an important biological marker for hepatobiliary diseases[3] and can react with prodrugs to give potent cancer inhibitors.[4] GGT has also been implicated in apoptosis inhibition[5,6] and in Parkinson's disease.[7] In the first step of its catalytic cycle, the enzyme is transiently acylated by a γ-glutamyl donor substrate. The acyl–enzyme intermediate then reacts with an acceptor substrate containing a free amino group (typically an amino acid or a peptide) to form a new isopeptide bond, regenerating the free enzyme through deacylation. The acyl–enzyme intermediate can also react with water to give glutamic acid and free enzyme. The catalytic cycle is proposed to follow a modified ping-pong mechanism.[3,8]

During our ongoing investigation of the detailed mechanism of action of rat kidney GGT, we sought an acyl donor substrate analogue that would be capable of rapidly acylating the free enzyme. Our initial attempts were centred around the use of simple *p*-nitrophenyl esters, which proved to be surprisingly inefficient acylating agents. Since L-γ-glutamyl-*p*-nitroanilide is widely used as a high affinity donor substrate analogue of glutathione (the in vivo substrate for GGT),[9] it seemed highly unlikely that the presence of the *p*-nitrophenyl group was problematic in the recognition of the esters examined. Our attention was thus focussed on the synthesis of a series of potential substrate analogues wherein the parent acid portion of the *p*-nitrophenyl ester was widely varied. Previous studies have shown that asparagine, *N*-acetyl-glutamine[10] and homoglutamine[11] do not act as donor substrates for GGT, but prior to this work few studies had been done on the importance of the ammonium and the carboxylate groups. Herein we thus report the synthesis and measurement of kinetic parameters for *p*-nitrophenyl esters of glutamic acid analogues wherein the ammonium and carboxylate groups have been modified. Kinetic parameters are also reported for the inhibition of the enzymatic reaction of the standard substrate L-γ-glutamyl-*p*-nitroanilide by the corresponding primary amides of the *p*-nitrophenyl esters studied. Taken together, these kinetic data permit a certain mapping of the γ-glutamyl donor substrate binding site of rat kidney GGT.

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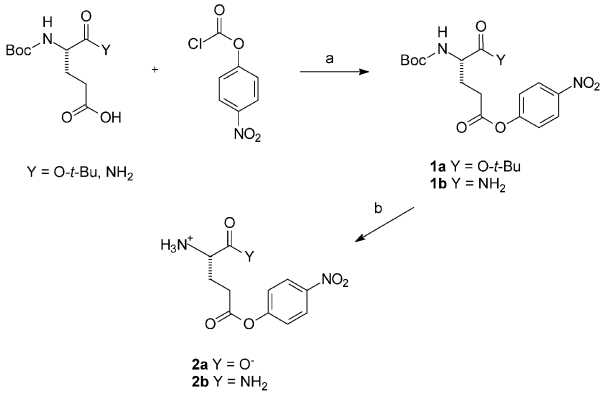
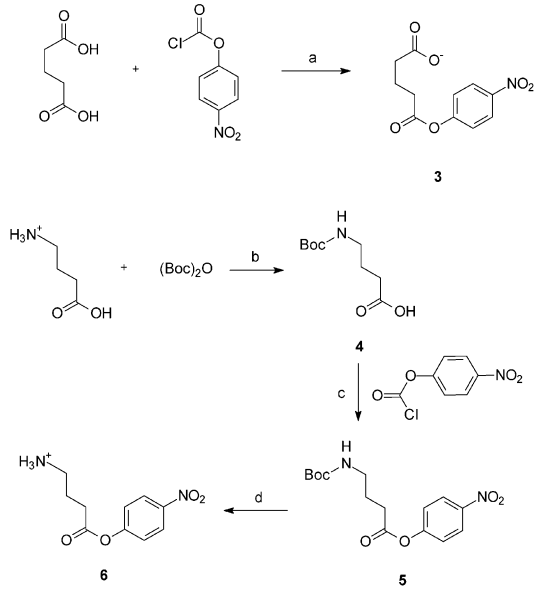
Results and Discussion

Synthesis of *p*-nitrophenyl activated esters and their amide derivatives

Although some of the donor substrate analogues tested were commercially available, most were synthesised as shown in Schemes 1 and 2 for the *p*-nitrophenyl activated esters and in Schemes 3 and 4 for the amide derivatives. As shown by the yields given in Schemes 1–4, the overall syntheses effected were for the most part efficient and straightforward. The method used to form the *p*-nitrophenyl ester group is noteworthy. We have found that one of the simplest methods is to combine the free carboxylic acid with *p*-nitrophenyl chloroformate in the presence of a base such as DMAP, according to a protocol similar to one published previously.[12] Removal of the Boc protecting groups of *p*-nitrophenyl ester compounds **1a** and **1b** (Scheme 1) must be carried out carefully and closely monitored to avoid cleavage of the ester groups. Recrystallization of **2b** was difficult and is responsible for the low yield obtained for this compound. In the synthesis of compound **3**, shown in Scheme 2, undesirable side products (such as the di-*p*-nitrophenyl ester of glutaric acid) decreased the yield of this reaction. As shown in Scheme 3, the corresponding amide derivatives of the *p*-nitrophenyl esters studied herein were generally obtained by ammoniolytic opening of glutaric anhydride to give **7**, or by displacing the *p*-nitrophenolate moiety of **5** to give **8**, which was nearly quantitatively deprotected to give **9**. As shown in Scheme 4, glutamine methyl ester (**11**) was synthesised from commercial Boc-L-glutamine by using diazomethane to afford the α-methyl ester **10** quantitatively, followed by quantitative Boc deprotection to give **11**.



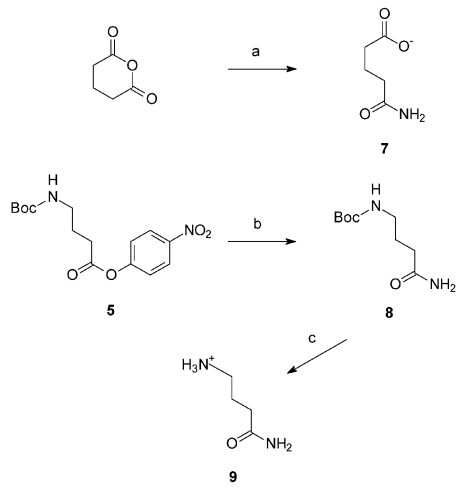
Scheme 1. Synthesis of L-γ-glutamyl-*p*-nitrophenyl ester **2a** and L-γ-isoglutaminyl-*p*-nitrophenyl ester **2b**: (a) 4,4-dimethylaminopyridine (DMAP), triethylamine, 25 °C, 75 min, ethyl acetate, 99% (for **1a**) or 165 min, acetonitrile, 71% (for **1b**); (b) gaseous HCl, 0–25 °C, 4 h, dichloromethane, 82% (for **2a**) or 22% (for **2b**).



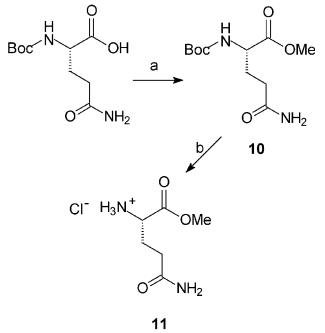
Scheme 2. Synthesis of *p*-nitrophenyl glutarate **3** and *p*-nitrophenyl 4-aminobutyrate **6**: (a) DMAP, triethylamine, acetonitrile, 33%; (b) triethylamine, 60 °C, methanol, 97%; (c) DMAP, triethylamine, acetonitrile, 95%; (d) gaseous HCl, 1 h, dichloromethane, 86%.

Several compounds containing a *p*-nitrophenyl moiety (*p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), **3**, **6**, **2a**, and **2b**) were used as donor substrates of GGT in the presence of a saturating concentration of glycylglycine, a good acceptor substrate. It was hoped

Kinetic studies with *p*-nitrophenyl esters



Scheme 3. Synthesis of 4-carboxybutyramide **7** and 4-aminobutyramide **9**: (a) gaseous ammonia, 4 days, dichloromethane, 34%; (b) gaseous ammonia, 1 day, dichloromethane, 91%; (c) gaseous HCl, 1 h, chloroform, 99%.



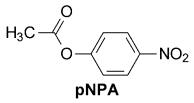
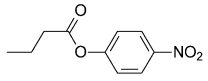
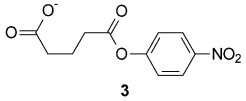
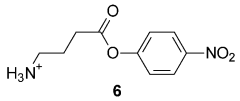
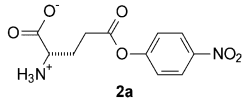
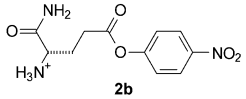
Scheme 4. Synthesis of glutamine methyl ester **10**: (a) diazomethane, diethyl ether/methanol (14:1), quantitative; (b) gaseous HCl, 1 h, chloroform, quantitative.

that by comparing the K_m values obtained for the different substrates studied, conclusions could be drawn regarding the necessity of the α-amino or carboxylate groups and the preferred length of the amino acid side chain. The results of these comparisons are shown in Table 1.

For pNPA, pNPB and **3**, no enzyme-catalyzed cleavage reaction was observed over the spontaneous background reaction. Apparently, these esters are not sufficiently recognized and bound by GGT to permit enzymatic catalysis, whereas their background reactions (with glycylglycine or with hydroxide ion) result in their efficient decomposition. For compounds **6**, **2a**, and **2b**, no cleavage reaction was observed during the time scale of the kinetic experiments. However, the initial absorbance values measured in these experiments suggests that the non-enzymatic background reactions are very rapid and already complete prior to addition of enzyme. The rapid disappearance of these compounds may be due to reaction with glycylglycine or hydroxide ion, or due to an intramolecular cyclization reaction following from the nucleophilic attack of the free α-amino group on the γ-carbonyl to give the corresponding lactams. For example, when compound **2a** was added to a D_2O solution (pD 7), subsequent analysis by ^1H NMR confirmed that pyroglutamic acid was rapidly formed with the liberation of *p*-nitrophenol.

For all of the *p*-nitrophenyl esters studied, their high background reactivity, relative to their enzymatic reactivity, prevented the determination of their K_m values. A different approach was therefore necessary in order to determine the relative importance of functional groups on the parent acid moiety for binding by GGT, as described below.

Table 1. Kinetic results obtained with *p*-nitrophenyl ester donor substrates

Compound tested	Kinetic result
 pNPA	No enzymatic reaction observed
 pNPB	No enzymatic reaction observed
 3	No enzymatic reaction observed
 6	No reaction observed
 2a	No reaction observed
 2b	No reaction observed

Kinetic studies with amide derivatives

The corresponding carboxamide derivatives of the *p*-nitrophenyl esters studied were either purchased or synthesised and tested as inhibitors of the colorimetric enzymatic reaction of the standard substrate L-γ-glutamyl-*p*-nitroanilide in the presence of the acceptor substrate glycylglycine. If the amide derivative serves as a donor substrate, participating in a non-colorimetric enzymatic reaction, the background colorimetric reaction will appear to be inhibited. This apparent inhibition would indicate that the amide must contain functional groups that are necessary for it to be recognized and bound as a potential substrate. This method provides an easy spectrophotometric fashion to measure quantitatively (although indirectly) the apparent affinity of GGT for different donor substrates. (However, it should be noted that when these amides serve as donor substrates for GGT, their apparent inhibition constants would take into account all forms of the enzyme with bound substrate along the reaction pathway. This would have the effect of lowering the observed K_i values relative to that of a simple competitive inhibitor.)

A graph typical of the inhibition of the standard transpeptidation reaction of L-γ-glutamyl-*p*-nitroanilide as a function of different concentrations of a carboxamide substrate as inhibitor is shown in Figure 1 for the results obtained with glutamine. In general, Michaelis–Menten and Lineweaver–Burk plots were used to determine the type of reversible inhibition observed for each amide compound studied. To determine the K_i value for each inhibitor, Dixon plots and/or secondary plots of the Lineweaver–Burk slopes were constructed. A typical example of the secondary plot is shown in Figure 2 for the results obtained with glutamine. The results obtained for all of the amide compounds tested are presented in Table 2.

It is interesting to note that acetamide and butyramide, the amide derivatives of pNPA and pNPB, are unable to act as inhibitors of the colorimetric enzymatic reaction of L-γ-glutamyl-*p*-nitroanilide. These active esters are frequently used to study various proteases, but apparently do not resemble the native substrate of GGT

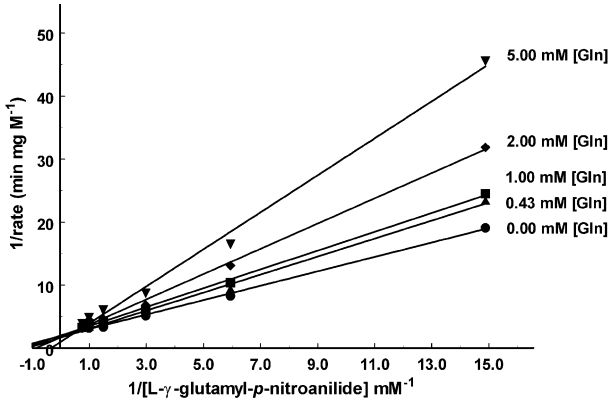
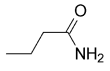
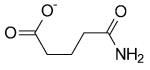
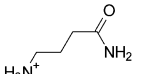
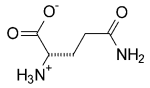
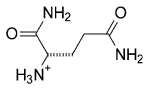
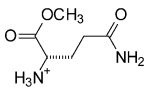
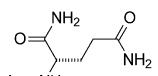


Figure 1. Lineweaver–Burk plots for the apparent inhibition of the colorimetric enzymatic reaction of L-γ-glutamyl-*p*-nitroanilide by glutamine. See Experimental for details.

closely enough to ensure their recognition. The inability of GGT to recognise pNPA as an acyl donor substrate has been previously documented.[13] Even the appropriate chain length of pNPB is insufficient to improve its affinity for the acyl donor substrate binding site of GGT. By contrast, it is not surprising that glutamine, whose parent acid group is identical to that of the native substrate(s) of GGT, has the lowest K_i value measured herein. Compound **3**, *p*-nitrophenyl glutarate, is not recognised by GGT as a donor substrate, owing in part to its instability under the kinetic reaction conditions, but its amide derivative **7** is a mixed non-competitive inhibitor with a K_i value of 12.6 mM. Since this mode of inhibition differs from that observed for glutamine, the K_i values cannot be compared directly, but it is noteworthy that they are of the same order of magnitude, suggesting that the carboxylate group of **7** significantly increases the affinity of this compound for GGT over that of butyramide. The fact that non-competitive inhibition was observed for **7** probably signifies that it is also able to compete for the acyl acceptor substrate binding site, since it also bears resemblance to glycylglycine.[14]

Compound **6** was also found to decompose rapidly and spontaneously, probably through an intramolecular lactamization reaction similar to that observed for **2a**. However, compound **9**, the amide derivative of **6**, was found to act as an uncompetitive inhibitor of the L-γ-glutamyl-*p*-nitroanilide reaction with a K_i value of 23.2 mM. In this case, it appears that the amide substrate competes more efficiently for the acyl *acceptor* substrate binding site rather than the acyl *donor* substrate binding site. This suggests that it resembles glycylglycine more strongly than it resembles a γ-glutamyl donor, and that the presence of either an amino group (**9**) or a carboxylate group (**7**) alone is not sufficient to ensure competitive inhibition at the donor substrate binding site. Three derivatives of glutamine were tested as inhibitors in order to determine the relative importance of the unmodified carboxylate and α-amino groups. As shown in Table 2 for glutamine amide, when the α-carboxylate moiety is replaced by a primary amide, the binding affinity is decreased relative to that of glutamine, but only by a factor of ~4, suggesting that this isosteric derivative is able to interact favourably with the donor substrate binding site. The decrease in affinity relative to glutamine may be due to the inability of glutamine amide to participate in electrostatic interactions. Previous mutagenesis studies have suggested that an arginine residue, which would be positively charged at physiological pH, may be present near the binding site to participate in such an electrostatic interaction to confer specificity for a free carboxylate group.[15] When the methyl ester of glutamine (**11**) was tested, it was also observed to be a competitive inhibitor with slightly less affinity than glutamine itself, once again indicating that electrostatic interactions may be important but are not absolutely essential. The fact that a sterically unimposing ester can be recognized and bound with reasonable affinity implies that other series of more synthetically accessible substrates and inhibitors could be prepared and tested in the future.

Table 2. K_i values and reversible inhibition type for amide derivative inhibitors of GGT-mediated γ-Glu-*p*-nitroanilide cleavage

Amide derivative compounds	K_i value (mM)	Inhibition type
	n/o[a]	—
	n/o[a]	—
 **7**	12.6	Mixed non-competitive
 **9**	23.2	Uncompetitive
	3.44	Competitive
	14.6	Competitive
	5.0	Competitive
	183[b]	Competitive

[a]No inhibition observed up to 100 mM of inhibitor.
[b]Approximate value extrapolated from Lineweaver–Burk plot.

When *N*-acetyl-L-glutamine was tested, it was found to be a very poor competitive inhibitor of the L-γ-glutamyl-*p*-nitroanilide reaction. This demonstrates the importance of an unsubstituted α-amino group, confirming previously obtained results that indicated that the α-amino group of the γ-glutamyl moiety must be unprotected.[10] Recognition of the α-amino group may also involve electrostatic attraction, and previous muta-

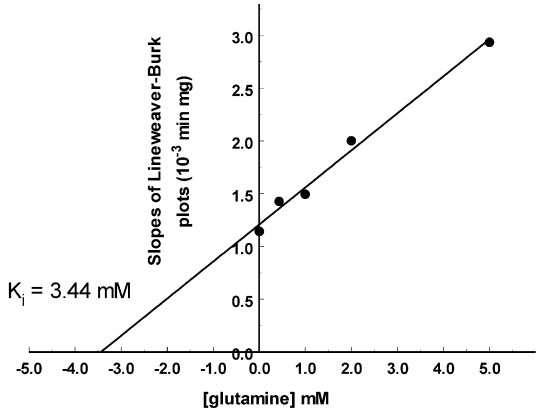


Figure 2. Secondary plot of Lineweaver–Burk slopes for the determination of the apparent K_i of glutamine inhibition of the colorimetric enzymatic reaction of L-γ-glutamyl-*p*-nitroanilide. See Experimental for details.

genesis studies[16] have suggested that a negatively charged aspartate residue may be near the binding site to play such a role. However, it has also been reported that *N*-methyl-glutamine, which would also exist in its zwitterionic form at neutral pH, does not serve as a donor substrate for GGT,[17] emphasizing the intolerance on the part of GGT of steric bulk at this position.

Conclusions

We synthesized compound **2a**, L-γ-glutamyl-*p*-nitrophenyl ester in order to use it as an acyl donor substrate in our ongoing kinetic studies of GGT. However, it proved to be inappropriate as a substrate due to its tendency to spontaneously form pyroglutamic acid under the enzyme kinetic reaction conditions. Furthermore, the protection of the α-amino group responsible for this background reaction essentially prevents the recognition of the compound by the enzyme. Further investigation herein of the structural requirements of the acyl donor substrate of GGT show that the enzyme displays a high degree of specificity not only with respect to chain length, as suggested elsewhere,[10] but also to the presence of free carboxylate and amino groups. This specificity is probably accomplished through the formation of a sterically restricted binding pocket that features an amino acid that is positively charged at physiological pH to stabilize the glutamyl carboxylate, and an amino acid that is negatively charged to stabilize the glutamyl ammonium group. The kinetic results presented herein confirm hypotheses previously purported based on mutagenesis experiments.[15,16]

Experimental

Materials and methods

Enzyme purification. Rat kidney GGT was purified according to an established protocol[3] that was slightly modified for the precipitation step. Briefly, during this step, ammonium sulphate was added to the enzyme solution to a concentration of 60% saturating $(NH_4)_2SO_4$, followed by centrifugation (18,000*g*, 30 min). Ammonium sulphate was then gradually added to the supernatant fraction to 90% saturating $(NH_4)_2SO_4$. The pellet resulting from centrifugation of this solution (18,000*g*, 30 min) was resuspended and purified by size exclusion chromatography, using a 62 cm×2.5 cm column of Bio-Gel A-0.5m (50–100 mesh) controlled by a Bio-Rad Econo-System. A volume of 350 mL of 0.05 M Tris–HCl buffer (pH 8.0) was used to elute the protein solution at 0.35 mL/min. After chromatography, fractions rich in activity were combined and frozen at −20 °C.

Synthesis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX300 (300 MHz) or a Bruker AMX400 (400 MHz). Solvents are indicated in the text and the chemical shifts are reported in ppm with internal reference to residual protonated solvent. Low and high resolution mass spectra were obtained from the Regional Centre of Mass Spectrometry at the Université de Montréal. Melting points were determined with a capillary tube Uni-melt Thomas-Hoover melting point apparatus and are reported as corrected values. All starting materials and solvents were obtained from Sigma-Aldrich.

***N*-Boc-L-glutamyl(γ-*p*-nitrophenyl ester) *t*-butyl ester (1a).**[18] *N*-Boc-L-glutamyl *t*-butyl ester (300 mg, 0.989 mmol) was added to 75 mL of ethyl acetate. Once completely dissolved, *p*-nitrophenyl chloroformate (219 mg, 1.09 mmol), triethylamine (152 μL, 1.09 mmol), and DMAP (12 mg, 0.10 mmol) were added. After stirring at room temperature for 75 min, the reaction was stopped and the solution was filtered. After evaporation of the filtrate, the solid residue was resuspended in ethyl acetate and extracted with half saturated $NaHCO_3$ until the aqueous phase was completely transparent (6×25 mL). The organic phases were combined and dried over $MgSO_4$. After evaporation, an oil was obtained (**1a**) (419 mg, 99%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 9H), 1.45 (s, 9H), 1.99 (m, 1H), 2.28 (m, 1H), 2.67 (td, J = 7.5 Hz, 7.5 Hz, 2H), 4.28 (m, 1H), 5.22 (d, J = 8.1 Hz, 1H), 7.28 (d, J = 8.9 Hz, 2H), 8.22 (dd, J = 0.5 Hz, 9.1 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.86, 28.18, 30.25, 53.01, 79.94, 82.39, 122.41, 125.03, 145.20, 155.31, 155.46, 170.40, 171.00. MS $(M+K)^+$ calcd 463.14828, obtained 463.14980.

***N*-Boc-L-γ-isoglutaminyl *p*-nitrophenyl ester (1b).** The same experimental protocol was followed as for **1a**, starting with Boc-L-γ-isoglutamine (200 mg, 0.811 mmol), using acetonitrile as solvent, and allowing the reaction to proceed for 3 h. A white product (**1b**) was thus obtained (212 mg, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (s, 9H), 2.05 (m, 1H), 2.30 (m, 1H), 2.79 (m, 2H), 4.33 (m, 1H), 5.28 (d, J = 7.1 Hz, 1H), 5.49 (br s, 1H), 6.15 (br s, 1H), 7.33 (d, J = 7.9 Hz, 2H), 8.30 (d, J = 7.9 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 28.38, 28.66, 31.37, 54.67, 80.78, 123.97, 126.04, 147.00, 157.00, 157.90, 171.50, 172.07. Mp 134–136 °C. MS $(M+H)^+$ calcd 368.14578, obtained 368.14510.

L-γ-Glutamyl *p*-nitrophenyl ester (2a). Compound **1a** (381 mg, 0.900 mmol) was dissolved in 10 mL of dichloromethane and the flask was set in an ice bath. Gaseous HCl generated from H_2SO_4 and NH_4Cl was bubbled through the solution for 1 h. The flask was then removed from the ice bath and warmed to room temperature for about 5 h, during which the reaction was followed by ^{1}H NMR. A white precipitate appeared. The reaction was stopped and the solvent was evaporated. A white solid (**2a**) was obtained (223 mg, 82%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.33 (m, 2H), 2.94 (td, J = 2.3 Hz, 7.5 Hz, 2H), 4.12 (t, J = 6.9 Hz, 1H), 7.40 (d, J = 9,3 Hz, 2H), 8.31 (d, J = 9,3 Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 26.33, 30.68, 52.94, 123.93, 126.15, 147.00, 156.84, 171.29, 171.54. Mp (141.0–142.5) °C. MS calcd 269.07736, obtained 269.07820.

L-γ-Isoglutaminyl *p*-nitrophenyl ester (2b). Compound **1b** (196 mg, 0.53 mmol) was dissolved in 5 mL of dichloromethane and gaseous HCl was generated as for

the preparation of **2a**. The flask was cooled in an ice bath for 30 min and then warmed to room temperature for 2 h. The solvent was evaporated and the residue was recristallized from methanol/ethyl acetate. A white solid (**2b**) was obtained (55 mg, 22%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.19 (m, 2H), 2.75 (t, $J=7.1$ Hz, 2H), 3.91 (t, $J=6.3$ Hz, 1H), 7.31 (d, $J=7.9$ Hz, 2H), 8.20 (d, $J=7.9$ Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.68, 30.31, 52.27, 124.28, 126.27, 146.08, 156.31, 170.87, 171.12. Mp 144 °C. MS calcd 268.09335, obtained 268.09460.

***p*-Nitrophenyl glutarate (3).** Glutaric acid (300 mg, 2.27 mmol) was dissolved in 30 mL acetonitrile. Then, triethylamine (0.23 g, 2.27 mmol), *p*-nitrophenyl chloroformate (503 mg, 2.50 mmol) and DMAP (28 mg, 0.23 mmol) were added in this order. The reaction was followed by thin layer chromatography (59:40:1 ethyl acetate/hexane/acetic acid). At the end of the reaction, the solvent was evaporated and the residue was dissolved in chloroform. This solution was washed with distilled water and the organic phase was partially evaporated and submitted to flash chromatography (60:40 ethyl acetate/hexane). The fractions containing the product were combined and evaporated. A yellow product (**3**) was obtained (47 mg, 33%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.11 (q, $J=7.2$ Hz, 2H), 2.56 (t, $J=7.1$ Hz, 2H), 2.79 (t, $J=7.3$ Hz, 2H), 7.30 (d, $J=9.3$ Hz, 2H), 8.29 (d, $J=9.3$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.68, 32.89, 33.34, 122.59, 125.44, 145.55, 155.45, 170.64, 178.96. Mp 92 °C. MS $(M+H)^+$ 254.1.

Boc-4-aminobutyric acid (4). 4-Aminobutyric acid (2.00 g, 19.4 mmol) was dissolved in 20 mL methanol. Then, triethylamine (11.8 g, 116.4 mmol) and di-*tert*-butyl dicarbonate (8.89 g, 40.7 mmol) were added and the flask was heated to 60 °C. The reaction was followed by TLC (99:1 ethyl acetate/acetic acid) and allowed to proceed overnight. The solvent was evaporated and the residual oil was dissolved in saturated sodium bicarbonate ($NaHCO_3$) and extracted with hexane (3×40 mL). The aqueous phase was acidified with 1 N HCl to pH 2–3. The aqueous phase was then extracted with ethyl acetate (3×30 mL) and the organic phases were combined and dried over $MgSO_4$. The solvent was evaporated and a white solid (**4**) was obtained (3.81 g, 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 1.83 (qu, $J=7.0$ Hz, 2H), 2.41 (t, $J=7.2$ Hz, 2H), 3.19 (m, 2H), 4.70 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.03, 28.27, 31.20, 39.69, 79.39, 156.12, 178.32. Mp 58 °C. MS $(M+H)^+$ calcd 204.12358, obtained 204.12300.

Boc-4-aminobutyryl-*p*-nitrophenyl ester (5). Compound **4** (3.80 g, 18.7 mmol) was dissolved in 75 mL acetonitrile. Triethylamine (2.08 g, 20.6 mmol), *p*-nitrophenyl chloroformate (4.15 g, 20.6 mmol) and DMAP (0.23 g, 1.87 mmol) were then added in this order. The reaction mixture was allowed to sit at room temperature overnight. The protonated triethylamine was filtered and discarded. Water was added to the filtrate and a yellow solid (**5**) appeared (5.72 g, 95%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H), 1.96 (q, $J=6.8$ Hz, 2H), 2.67 (t, $J=7.1$ Hz, 2H), 3.28 (m, 2H), 4.63 (br s, 1H), 7.31 (d, $J=9.3$ Hz, 2H), 8.29 (d, $J=9.3$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.36, 28.54, 31.62, 39.74, 79.62, 122.62, 125.35, 145.47, 155.53, 156.31, 171.02. Mp 120 °C. MS $(M+H)^+$ calcd 325.13995, obtained 325.13890.

4-Aminobutyryl-*p*-nitrophenyl ester (6). Compound **5** (300 mg, 0.93 mmol) was dissolved in 20 mL of dichloromethane. Gaseous HCl, generated by sulphuric acid (H_2SO_4) and ammonium chloride (NH_4Cl), was bubbled through the reaction mixture for 1 h. The solution was filtered and a yellow solid (**6**) was obtained (208 mg, 86%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.05 (q, $J=7.1$ Hz, 2H), 2.82 (t, $J=7.1$ Hz, 2H), 3.06 (t, $J=7.7$ Hz, 2H), 7.39 (d, $J=9.2$ Hz, 2H), 8.31 (d, $J=9.2$ Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 23.50, 31.55, 39.88, 123.89, 126.16, 146.90, 156.86, 171.82. Mp 149–151 °C. MS (M^+) 225.2.

4-Carboxybutyramide (7). Glutaric anhydride (500 mg, 4.38 mmol) was dissolved in 10 mL dichloromethane. Gaseous ammonia (NH_3), generated by sodium hydroxide (NH_4OH) and ammonium chloride (NH_4Cl), was bubbled through the reaction mixture until completion of the reaction (4 days). The reaction mixture was filtered and the solid thus recovered was washed with 3×10 mL of dichloromethane. A white solid (**7**) was obtained (190 mg, 34%). ^{1}H NMR (300 MHz, CD_3OD) δ 1.88 (q, $J=7.3$ Hz, 2H), 2.25 (t, $J=7.8$ Hz, 2H), 2.33 (t, $J=7.4$ Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 22.12, 34.09, 35.47, 176.86, 178.34. Mp 89–90 °C. MS $(M+H)^+$ 132.1.

Boc-4-aminobutyramide (8). Compound **5** (350 mg, 1.08 mmol) was dissolved in 20 mL of dichloromethane. Gaseous ammonia, generated as for the preparation of **7**, was bubbled through the reaction mixture until completion of the reaction (1 day). The reaction was followed by TLC (99:1 ethyl acetate/acetic acid). The solvent was evaporated and the residue was dissolved in a minimum of chloroform. Flash chromatography (100% ethyl acetate) was performed and the fractions containing the product were combined and evaporated. The residue was washed with *n*-propanol and dried in vacuo overnight. A yellow solid (**8**) was obtained (198 mg, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 9H), 1.81 (q, $J=6.5$ Hz, 2H), 2.28 (t, $J=7.1$ Hz, 2H), 3.19 (t, $J=6.4$ Hz, 2H), 4.88 (br s, 1H), 5.84 (br s, 1H), 6.41 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.22, 28.31, 32.71, 39.60, 79.43, 156.54, 175.41. Mp 125 °C. MS $(M+H)^+$ calcd 203.13957, obtained 203.13870.

4-Aminobutyramide (9). Compound **8** (130 mg, 0.640 mmol) was dissolved in 10 mL chloroform. Gaseous HCl, generated as for the synthesis of **6**, was bubbled through the reaction mixture for 1 h and allowed to stir overnight. The reaction was filtered and a yellow solid (**9**) was obtained (89 mg, 100%). ^{1}H NMR (300 MHz, CD_3OD) δ 1.90 (q, $J=7.2$ Hz, 2H), 2.37 (t, $J=7.1$ Hz, 2H), 2.96 (t, $J=7.3$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 24.29, 32.87, 40.29, 178.02. Mp 98–100 °C. MS $(M+H)^+$ calcd 103.08714, obtained 103.08680.

***N*-Boc-L-glutamine methyl ester (10).** *N*-Boc-L-glutamine (1.135 g, 6.95 mmol) was dissolved in 20 mL diethyl ether/methanol (14:1). A solution of diazomethane in diethyl ether (prepared from Diazald®) was added until persistence of the yellow coloration. The solvent was evaporated. A white solid was obtained (1.198 g, quantitative). ^{1}H NMR (300 MHz, CD_3OD) δ 1.44 (s, 9H), 1.91 (m, 1H), 2.10 (m, 1H), 2.32 (t, $J=7.5$ Hz, 2H), 3.73 (s, 3H), 4.14 (t, $J=8.5$ Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 28.30, 28.69, 32.48, 52.69, 54.52, 80.51, 157.85, 174.36, 177.40. Mp (183–185)°C. MS $(M+H)^+$ calcd 261.14505, obtained 261.14579.

L-Glutamine methyl ester (11). Compound **10** (0.560 g, 2.15 mmol) was dissolved in 10 mL chloroform. Gaseous HCl was generated as for the preparation of **2a**. The flask was cooled in an ice bath for 1 h and then warmed to room temperature for 1 h supplementary. The solvent was evaporated and viscous coloured oil was obtained (0.419 mg, quantitative). ^{1}H NMR (300 MHz, D_2O) δ 2.12 (m, 2H), 2.41 (m, 2H), 3.74 (s, 1H), 4.07 (t, $J=7.8$ Hz, 1H). ^{13}C NMR (100 MHz, D_2O) δ 25.55, 30.58, 52.47, 53.86, 170.38, 171.13 MS $(M+H)^+$ 162.1.

Kinetics

For the kinetic studies, absorbance values were measured either on a Pharmacia Biotech Ultrospec 2000 or on a Cary 100 Bio spectrophotometer. All enzyme kinetic data were analyzed using curve-fitting software Axum 4.0, according to the formula $v=(V_{max}*\mathrm{Sub})/(K_m+\mathrm{Sub})$, where v is the initial velocity, V_{max} is the maximal velocity, Sub is the substrate concentration and K_m is the Michaelis constant.

***p*-Nitrophenyl ester substrates.** All the organic compounds containing a *p*-nitrophenyl ester moiety were dissolved in organic solvents. The compound *p*-nitrophenyl acetate was dissolved in acetonitrile, *p*-nitrophenyl butyrate in 95% ethanol, *p*-nitrophenyl glutarate, *p*-nitrophenyl 4-aminobutyrate and L-γ-isoglutamyl-*p*-nitrophenyl ester in methanol and L-γ-glutamyl-*p*-nitrophenyl ester in DMF, in order to make 0.05 M stock solutions. All kinetics were performed using 20 mM glycylglycine as an acceptor substrate, different concentrations of the donor substrate (between 500 and 1500 μM) and 0.1 M MOPS buffer at pH 7.0, for a final volume of 1 mL. Reactions were initiated by adding 0.051 U GGT. The release of *p*-nitrophenolate was followed spectrophotometrically at 402 nm. After 10 min, the slope of the graph of absorbance versus time was measured and converted to velocity by dividing by the extinction coefficient ($\varepsilon=8470\ M^{-1}\ cm^{-1}$ determined at pH 7.0). Corrections were made for the effect of organic solvent on the activity of the enzyme, determined from activity assays performed under the same conditions.

Amide derivative substrates. Using 0.1 M MOPS pH 7.0 buffer as a solvent, stock solutions of acetamide (0.100 M), butyramide (0.200 M), glutamine (0.025 M) glutamine amide (0.100 M) and glutamine methyl ester (0.500 M) were prepared. The compounds 4-carboxybutyramide and 4-aminobutyramide were dissolved in methanol to give stock solutions of 0.255 and 0.400 M, respectively. A stock solution of 0.156 M of *N*-acetylglutamine was made in 19% acetonitrile–MOPS buffer. Because it is better that no more than 10% of organic solvent is present in the enzymatic reaction, the low solubility of the last three compounds limited the concentrations at which they could be tested. All of the kinetic studies were carried out using different concentrations of L-γ-glutamyl-*p*-nitroanilide (between 50 and 1680 μM), 20 mM glycylglycine, a fixed concentration of amide substrate (usually between 0 and 100 mM when possible) and 0.1 M MOPS pH 7.0 buffer in a final volume of 1 mL. Reactions were initiated by adding 3.38 mU of GGT. The liberation of *p*-nitroaniline was followed spectrophotometrically at 410 nm. An extinction coefficient value of $9200\ M^{-1}\ cm^{-1}$ at pH 7.0 was used for calculating initial rates.[19] The K_i values for the apparent inhibition of the colorimetric enzymatic reaction of L-γ-glutamyl-*p*-nitroanilide due to the non-colorimetric enzymatic reaction of each amide substrate were determined by Lineweaver–Burk and/or Dixon plots. Values of K_m and V_{max} were determined as for the *p*-nitrophenyl esters.

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