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Synthesis of Aza and Oxaglutamyl-*p*-nitroanilide Derivatives and Their Kinetic Studies with γ-Glutamyltranspeptidase

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Abstract—A new series of L-glutamic acid *p*-nitroanilide analogues has been synthesized and tested as substrates and inhibitors of rat kidney γ-glutamyltranspeptidase (GGT). Kinetic parameters (K_m and k_{cat}) were determined for each analogue and provide insight into the scope and limits of GGT catalytic efficiency.

γ-Glutamyltranspeptidase (GGT; EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme. It has been implicated in many physiological disorders, including Parkinson's disease[1] and perturbation of apoptosis.[2,3] GGT catalyses the transfer of a γ-glutamyl moiety from the in vivo donor substrate, glutathione (GSH), to an active site nucleophile to form an acyl-enzyme intermediate during the acylation step of its catalytic cycle. This intermediate can then react, during the deacylation step, with an acceptor substrate containing a free amino group (normally an α-amino acid) to form a new γ-glutamyl isopeptide bond and regenerate the free enzyme. In the absence of a primary amine acceptor substrate, the acyl enzyme reacts slowly with water, giving a ping pong catalytic cycle that is modified to include hydrolytic deacylation.[4]

Rat kidney is a rich source of GGT that is particularly widely used.[4] Although rat kidney GGT has been studied by our group[5,6] and many others,[7] its three-dimensional structure and many of the details of its catalytic mechanism remain unknown. For example, the active site nucleophile (possibly a threonine hydroxyl group) and general acid/base have not been unambiguously identified.[6] Our ongoing studies of the mechanism of GGT action and inhibition have been based on kinetic studies of the enzymatic reactions of different synthetic donor and acceptor substrate analogues.

Recently, various GSH analogues have been synthesized that contain different heteroatoms at the *gamma* position adjacent to the carbonyl of the 'glutamyl' side chain. These compounds were tested as inhibitors of hog kidney GGT.[8–10] However, the effects of the heteroatoms on the strength of the C=ONH bond and the implications on the mechanism of GGT were not discussed. In order to probe the nucleophilicity of GGT and provide details of the mechanism of the acylation and deacylation steps, we decided to synthesize similar GGT donor substrate analogues, but containing an activated and easily detectable leaving group. L-Glutamic acid γ-*p*-nitroanilide (**L-GPNA**) is well known to be comparable to GSH as a donor substrate,[4] so analogues of this compound containing heteroatoms on the side chain (–NH–CO–NH–Ar, –O–CO–NH–Ar) were synthesized (Fig. 1). We prepared the methyl esters as well as the free carboxylic acids in order to test the effect of this substitution on enzyme affinity. The synthesis of these glutamine analogues and the results of their kinetic evaluation are reported herein.

Scheme 1 depicts the synthesis of L-glutamic acid γ-*p*-nitroanilide methyl ester (**4**) from L-glutamic acid.

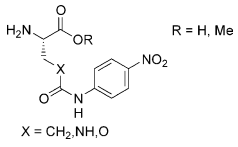

Figure 1. L-γ-Glutamic acid *p*-nitroanilide analogues studied herein.

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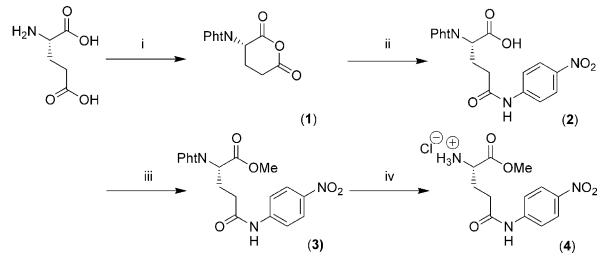
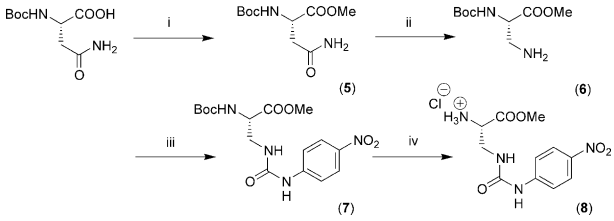


Scheme 1. Synthesis of L-glutamic acid γ-*p*-nitroanilide methyl ester **4**: (i) (a) phthalic anhydride at 180 °C; (b) acetic anhydride, 54% (two steps); (ii) *p*-nitroaniline, acetic acid, benzene at 65 °C, 100%; (iii) ethereal diazomethane, 100%; (iv) (a) hydrazine, MeOH, rt; (b) HCl (1 N), 43%.

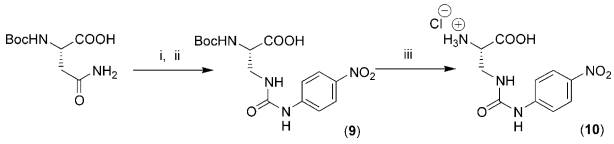
The first two steps to give the phthalyl derivative **2** are known.[11] The methyl ester **3** is subsequently prepared by reaction with an ethereal diazomethane solution. Final deprotection of the phthalyl group using hydrazine in the presence of the methyl ester gave a modest yield, typical for this reaction.

Schemes 2 and 3 outline the synthesis of the L-4-aza derivatives **8** and **10** from Boc-L-asparagine. The key amine **6** was obtained from a Hofmann rearrangement[12] of an appropriately protected L-asparagine methyl ester. In this case, this rearrangement was effected using a trivalent iodide reagent.[13] The amine was then condensed with *p*-nitrophenyl isocyanate (PNI). Routine removal of the Boc protecting group with TFA gave the aza derivative methyl ester **8**.

As shown in Scheme 3, compound **10** was obtained in a similar fashion by a Hofmann rearrangement of the free acid to give the corresponding free amine, followed directly by its condensation to give **9**, without requiring protection of the carboxylate group. Attempts to isolate the free amine resulted in lower yields. The Boc deprotection gives the desired compound **10**, quantitatively.



Scheme 2. Synthesis of L-4-azaglutamic acid γ-*p*-nitroanilide methyl ester **8**: (i) ethereal diazomethane, 94%; (ii) $PhI(O_2CCF_3)_2$, pyridine, DMF/H_2O, 87%; (iii) *p*-nitrophenyl isocyanate, THF/CH_2Cl_2, 56%; (iv) (a) TFA, anisole, CH_2Cl_2 at 0 °C; (b) HCl (0.1 N), 91%.



Scheme 3. Synthesis of L-4-azaglutamic acid γ-*p*-nitroanilide **10**: (i) $PhI(O_2CCF_3)_2$, pyridine, DMF/H_2O; (ii) *p*-nitrophenyl isocyanate, 56% (two steps); (iii) (a) TFA, anisole, CH_2Cl_2 at 0 °C; (b) HCl (0.1 N), 81%.

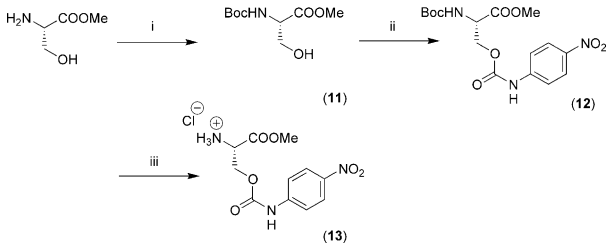
Scheme 4 outlines the synthesis of L-4-oxaglutamyl γ-*p*-nitroanilide methyl ester **13** from L-serine methyl ester. Initially the free α-amino group was protected as a Boc derivative **11**. Subsequent condensation of the side chain alcohol of the serine derivative with PNI was carried out in the presence of pyridine to reduce reaction times.

The free acid derivative of methyl ester **13**, L-4-oxaglutamic acid γ-*p*-nitroanilide, was not synthesized, due to its inherent instability resulting in the rapid liberation of *p*-nitroaniline.

All synthetic reactions were followed by TLC and the synthetic intermediates were characterized by ^1H and ^{13}C NMR and MS. Compounds **4**,[14] **8**,[15] **10**,[16] and **13**[17] were selected for kinetic evaluation. **L-GPNA** was synthesised as previously described.[11]

GGT was purified from rat kidney as previously described.[6] For each test substrate, 15 mM stock solutions were prepared in 0.1 M Tris–HCl pH 8.0 buffer. When compounds were tested as donor substrates, a concentration range of 168–2000 μM of the compound was used in the presence of 20 mM of glycylglycine as acceptor substrate in Tris–HCl buffer. Reactions were initiated by the addition of 3.38 mU of GGT. Liberated *p*-nitroaniline was detected spectrophotometrically at 410 nm ($\varepsilon = 8800\ M^{-1}\ cm^{-1}$)[4] on a Cary 100 Bio spectrophotometer. When compounds were tested as acceptor substrates, a high concentration of 1 mM **D-GPNA** (to avoid autotranspeptidation)[18,19] was used as donor substrate and concentrations of 1–8 mM of the compound of interest were studied under the same conditions as for the donor substrate experiments. For compound **13**, the experiments were done in 0.1 M Mops pH 7.0 buffer due to the instability of the substrate at pH 8.0. For inhibition studies, different concentrations (168–1400 μM) of **L-GPNA** and a saturating concentration (20 mM)[4] of glycylglycine were used in the presence of constant concentrations (100–1200 μM) of compound **8** or **10**. K_M, k_{cat} and K_i values were determined by Michaelis–Menten, Lineweaver–Burk, or Dixon plots analyzed by Axum 4.0 curve-fitting software. The k_{cat} values thus obtained were normalized to a specific activity of 837 U/mg. The results from these tests are reported in Table 1.

We have determined kinetic constants for **L-GPNA**, as a donor and an acceptor substrate, that are comparable



Scheme 4. Synthesis of L-4-oxaglutamic acid γ-*p*-nitroanilide methyl ester **13**: (i) $(Boc)_2O$, Et_3N, MeOH at 0 °C, 68%; (ii) *p*-nitrophenyl isocyanate, pyridine, THF/CH_2Cl_2, 59%; (iii) (a) TFA, anisole, CH_2Cl_2 at 0 °C; (b) HCl (0.1 N), 88%.

Table 1. Kinetic results of the compounds **L-GPNA**, **4**, **8**, **13** and L-serine methyl ester acting as donor and/or acceptor substrates for GGT-mediated transpeptidation

Compd	Nature of substrate	K_M (mM)	k_{cat} (10^3 min^{-1})	k_{cat}/K_M (10^6 min^{-1} M^{-1})
L-GPNA	Donor	0.47±0.05	69±7	150±20
	Acceptor	6.3±0.2	16±2	2.5±0.1
4	Donor	5.7±0.5	1.5±0.3	0.27±0.02
	Acceptor	6±1	3.2±0.4	0.58±0.07
8	Donor	Inactive[a]	Inactive[a]	Inactive[a]
	Acceptor	4±1	6.1±0.6	1.4±0.1
10	Donor	Inactive[a]	Inactive[a]	Inactive[a]
	Acceptor	Insoluble[b]	Insoluble[b]	Insoluble[b]
13[c]	Donor	Inactive[a]	Inactive[a]	Inactive[a]
	Acceptor	1.5±0.1	4.8±0.4	3.0±0.3
L-Serine methyl ester	Donor	N/A[d]	N/A[d]	N/A[d]
	Acceptor	34±9	1.3±0.2	0.038±0.006

[a]These compounds were not active at 2 mM with 33.8 mU of GGT.
[b]Insoluble for concentrations greater than 15 mM.
[c]This compound was tested at pH 7.0, 0.1 M Mops buffer.
[d]N/A, not applicable.

to values previously obtained.[6,20] It is interesting to note that compound **4**, the methyl ester of **L-GPNA**, is also recognized by the enzyme and is able to serve as a donor substrate, although less efficiently.

Surprisingly, our results clearly indicate that the presence of heteroatoms in the γ-position of the side chain of L-glutamic acid γ-*p*-nitroanilide analogues **8** and **13** prevents these analogues from serving as donor substrates. Although the attenuated reactivity of urea and carbamate derivatives with respect to their analogous carboxamide compounds has been well documented in the literature,[21] these derivatives are typically capable of serving as donor substrates. Their modified reactivity has thus often been exploited in the design of compounds that may be able to 'acylate' a given enzyme to form a covalent intermediate that is resistant to subsequent nucleophilic attack, effectively irreversibly inhibiting the enzyme.[22] However, in this case, contrary to our expectations, GGT is apparently unable to catalyze even the initial 'acylation' from **8** or **13**. Hence, these compounds only show activity as acceptor substrates, as do most amino acids.[23]

As a control experiment to ensure that the methyl ester groups of compounds **8** and **13** were not responsible for their inefficacy as donor substrates, compound **10** was synthesized and tested. A free acid derivative of urea **8**, it was also found to be incapable of serving as a donor substrate.

Finally, it was important to confirm that the inactivity of compounds **8**, **10**, and **13** was due to their attenuated reactivity, and not simply low affinity for the donor substrate binding site of GGT. Competition experiments were thus performed between **L-GPNA** and compound **10** in the presence of a saturating concentration of glycylglycine as acceptor substrate. It was found that **10** acts as a mixed non-competitive inhibitor under these conditions [$K_{i,comp} = (3.8 \pm 0.1)$ mM, $K_{i',uncomp} = (1.7 \pm 0.2)$ mM, Fig. 2]. This indicates that **10** is bound in both the donor substrate binding site and elsewhere on the enzyme, probably in the acceptor substrate binding site, as is commonly observed for such a two substrate enzyme that follows a ping-pong mechanism.[4,7] Inhibition studies with compound **8** led to the same conclusions. Thus, the methyl esters of the heteroatomic analogues are bound by the enzyme but do not react as donor substrates.

Compounds **4**, **8**, and **13** were then studied in more detail in their capacity as acceptor substrates. Glycylglycine, the best acceptor substrate for GGT, has a k_{cat}/K_M of 5.9×10^6 min^{-1} M^{-1},[23] but L-serine methyl ester was found to have a value of 3.8×10^4 min^{-1} M^{-1}. As shown in Table 1, our heteroatomic analogue compounds are more efficient acceptor substrates than simple amino acids, but are still less efficient than certain dipeptides. The enhanced efficiency (k_{cat}/K_M) of our compounds, compared to L-serine methyl ester, could possibly be due to the formation of hydrogen bonds between the substrate binding site and the side-chain carbonyl or the heteroatom at the γ-position. Although there may be a positive correlation of their efficiency with the electronegativity of the atom at the γ-position,

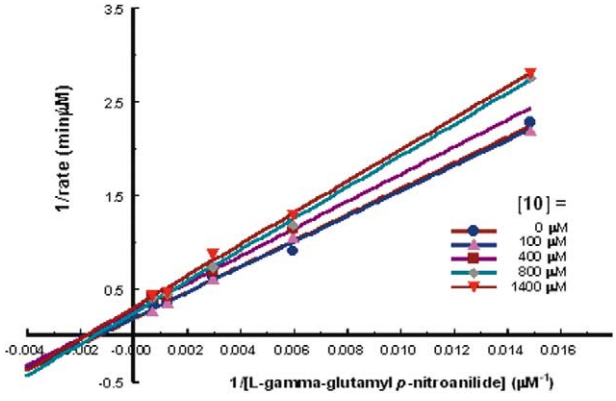


Figure 2. Lineweaver–Burk plot for the mixed non-competitive inhibition of **L-GPNA** by **10** (see text for details).

modelling studies have provided no reason to believe the heteroatomic analogues adopt any unusual conformation that may improve their affinity or reactivity as acceptor substrates, for example, through the formation of an intramolecular α-amino to γ-hydrogen bond. Perhaps their improved efficiency is due to their capacity to be bound in an orientation similar to that of a (γ-)dipeptide, thereby resembling the efficient dipeptide substrate glycylglycine.

It also appears that the methyl ester group has an adverse effect on acceptor substrate efficiency, as shown in the comparison of **L-GPNA** and **4**. A similar comparison between L-serine ($k_{cat}/K_M = 0.138 \times 10^6$ min^{-1} M^{-1})[23] and its methyl ester (Table 1) confirms this pattern. The corresponding comparison of compounds **8** and **10** was impossible, since the low solubility of compound **10** at pH 8.0 made it impractical to determine its efficiency precisely.

In summary, a new class of compounds containing heteroatoms at the γ-position of the side chain of a glutamyl moiety has been synthesized successfully. Although compounds **8**, **10**, and **13** contained a good leaving group compared to previous heteroatomic glutathione analogues,[8–10] their urethane and carbamate bonds were found to be resistant to cleavage by purified rat kidney GGT. The decreased electrophilicity of the carbonyl group in these compounds and the poor nucleophilicity of the active site (threonine) nucleophile of GGT could explain this lack of reactivity. Nevertheless, this class of compounds should prove useful for enzymes containing a stronger active site nucleophile, as is the case, for example, for transglutaminase[24] and the cysteine proteases.

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14. (**4**): Yellow solid; $[\alpha]_D$ +13.3° (*c* 1, MeOH); ^1H NMR (D_2O, 400 MHz) δ 2.22 (m, 2H), 2.62 (m, 2H), 3.72 (s, 3H), 4.14 (t, 1H), 7.59 (d, $J = 9.1$ Hz, 2H), 7.70 (d, $J = 9.2$ Hz, 2H); ^{13}C NMR (CD_3OD, 75 MHz) δ 26.5, 33.0, 53.4, 54.6, 120.3, 125.8, 144.5, 146.0, 170.6, 172.6; *m/z* 281.1096 (MH^+, $C_{12}H_{15}N_3O_5$ requires 281.1012).
15. (**8**): Yellow solid; $[\alpha]_D$ −26.1° (*c* 1, MeOH); ^1H NMR (D_2O, 300 MHz) δ 3.79 (t, $J = 4.3$ Hz, 2H), 3.84 (s, 3H), 4.30 (t, 1H), 7.52 (d, $J = 9.2$ Hz, 2H), 8.20 (d, $J = 9.2$ Hz, 2H); ^{13}C NMR (D_2O, 75 MHz) δ 39.7, 53.7, 54.1, 118.5, 125.5, 142.1, 145.6, 157.0, 169.3; *m/z* 282.0969 (MH^+, $C_{11}H_{14}N_4O_5$ requires 282.0964).
16. (**10**): Yellow solid; $[\alpha]_D$ −27.7° (*c* 0.6, MeOH); ^1H NMR (CD_3OD, 300 MHz) δ 3.58 (br d, $J = 14.1$ Hz, 1H) 3.76 (br d, $J = 14.3$ Hz, 1H), 4.19 (t, 1H), 7.65 (d, $J = 8.8$ Hz, 2H), 8.12 (d, $J = 8.7$ Hz, 2H); ^{13}C NMR (CD_3OD, 75 MHz) δ 40.9, 54.0, 118.7, 125.8, 143.1, 147.3, 157.6, 169.9; *m/z* 283.1.
17. (**13**): Yellow solid; $[\alpha]_D$ −16.0° (*c* 1, MeOH); ^1H NMR (D_2O, 400 MHz) δ 3.88 (s, 3H), 4.58 (t, 1H), 4.66 (dd, $J = 12.5$ Hz, $J = 3.6$ Hz, 1H), 4.74 (dd, $J = 12.4$ Hz, $J = 4.4$ Hz, 1H), 7.52 (d, $J = 9.1$ Hz, 2H), 8.18 (d, $J = 9.1$ Hz, 2H); ^{13}C NMR (D_2O, 75 MHz) δ 52.7, 54.3, 62.8, 118.9, 125.5, 143.2, 144.3, 153.8, 168.3; *m/z* MH^+ 283.9.
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