γ-Glutamyl transpeptidase acylation with peptidic substrates: free energy relationships measured by an HPLC kinetic assay†

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γ-Glutamyl transpeptidase (GGT, EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme linked to the external cellular membrane that catalyzes the hydrolysis of glutathione as well as the transfer of its γ-glutamyl group to amino acids and dipeptides in a transpeptidation reaction. The measurement of both the hydrolytic and transpeptidation activity of this important enzyme is a challenge, since its native substrates are not highly chromogenic. We have developed an HPLC-based method for the quantitative photometric detection of numerous enzyme substrates and products, after their pre-column derivation with dabsyl chloride. The broad applicability of this method was demonstrated in the kinetic investigation of transpeptidation reactions of rat kidney GGT with glutathione, its native substrate, as well as a series of pertinent glutathione analogues. The pH-rate profile constructed for glutathione confirmed the dependence on the ionisation state of at least two residues. Analysis of the free-energy relationships in the series of synthetic peptidic substrate analogues revealed the importance of enzyme–substrate interactions unrelated to amine leaving group basicity during the acylation step. These results are further interpreted in the context of the recently published structure for a similar GGT.

Introduction

γ-Glutamyl transpeptidase (GGT, EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme linked to the external surface of cells through a membrane anchor found in its large ~46 kDa subunit, that is bound non-covalently to its small ~22 kDa subunit.[1] GGT plays roles in several biological processes, including the biosynthesis of leukotrienes D4,[2] cellular detoxification through the formation of mercapturic acids,[3] homeostasis of glutathione (GSH), its *in vivo* substrate, and possibly the transport of amino acids across the cellular membrane *via* the γ-glutamyl cycle.[4] This enzyme has also been implicated in many physiological disorders such as Parkinson's disease,[5] inhibition of apoptosis,[6] diabetes[7] and asthma.[2,8] It is particularly concentrated in the kidney, brain and pancreas.[1]

GGT catalyzes three different types of reactions *via* a modified ping-pong mechanism.[9,10] The first reaction, transpeptidation, comprises the transient transfer of a γ-glutamyl moiety from a donor substrate, GSH, to form an intermediate acyl-enzyme, followed by acyl transfer to an acceptor substrate—either an amino acid or a dipeptide—to yield a γ-glutamyl peptide derivative.[1,11–13] At higher concentrations, GSH may react as both donor and acceptor substrates, leading to autotranspeptidation, the second type of catalytic reaction.[9] The third catalytic reaction is hydrolysis, resulting in the formation of glutamic acid when a water molecule acts as an acceptor substrate.[9,10,14]

In the past, several mechanistic studies have been carried out with GGT,[1,11,15–18] but much remains to be learned concerning the catalytic mechanism and the specific amino acid residues involved. A better comprehension of this mechanism will be important for the development of more efficient inhibitors for the treatment of certain disorders.[2,5–8] Until recently, the mechanism of GGT was studied primarily using chromogenic substrate analogues such as γ-glutamyl-*p*-nitroanilide, from which the intensely yellow-coloured *p*-nitroaniline is liberated as a reaction product.[1,13,15,19] We have also studied other *para*-substituted γ-glutamyl anilide substrate analogues through the colorimetric determination of released aniline by a discontinuous diazotization assay.[15] These studies have led to a greater understanding of the mechanism of GGT acylation, but the relevance of these studies with respect to the mechanism of the native enzyme reaction may be questioned, considering the structural differences between a γ-glutamyl anilide and glutathione, the native substrate. However, the study of more structurally similar, peptidic substrate analogues has been limited by the lack of a convenient kinetic method for following their reaction with GGT. A high-performance liquid chromatography (HPLC)-based method has been reported[20] for the specific detection of glutamate deriving from the hydrolase activity of GGT, but this method is not applicable to the study of physiologically relevant GGT-mediated transpeptidation reactions. In this paper, we report the development of a complementary HPLC-based method for the quantitative photometric detection of numerous primary amine products of the acylation step, after their pre-column derivation with (dimethylamino)azobenzenesulfonyl chloride (dabsyl chloride, DABS-Cl).

This method permitted the study of rat kidney GGT-mediated transpeptidation reactions from peptidic GSH substrate analogues whose products cannot be detected simply and directly by spectrophotometry (Scheme 1). A series of such analogues was

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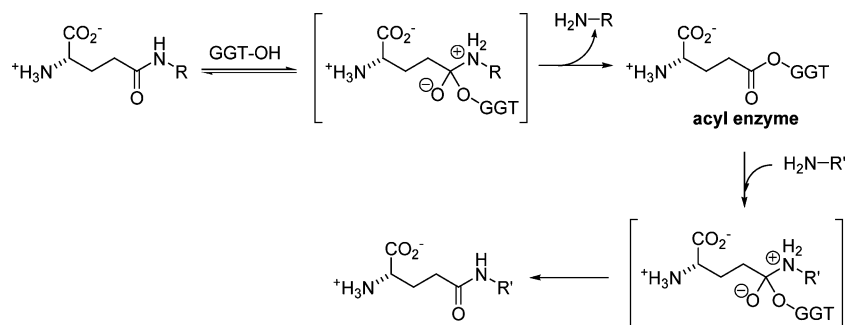
Scheme 1 Simplified mechanism of transpeptidation reactions mediated by GGT.

synthesized and used to investigate the free-energy relationships and pH-rate effects of the acylation step of the catalytic transpeptidation process. Furthermore, the recent publication[21] of the first high-resolution structure of GGT allowed, for the first time, the interpretation of the mechanistic consequences of the results of our studies in the context of a model for the enzyme structure.
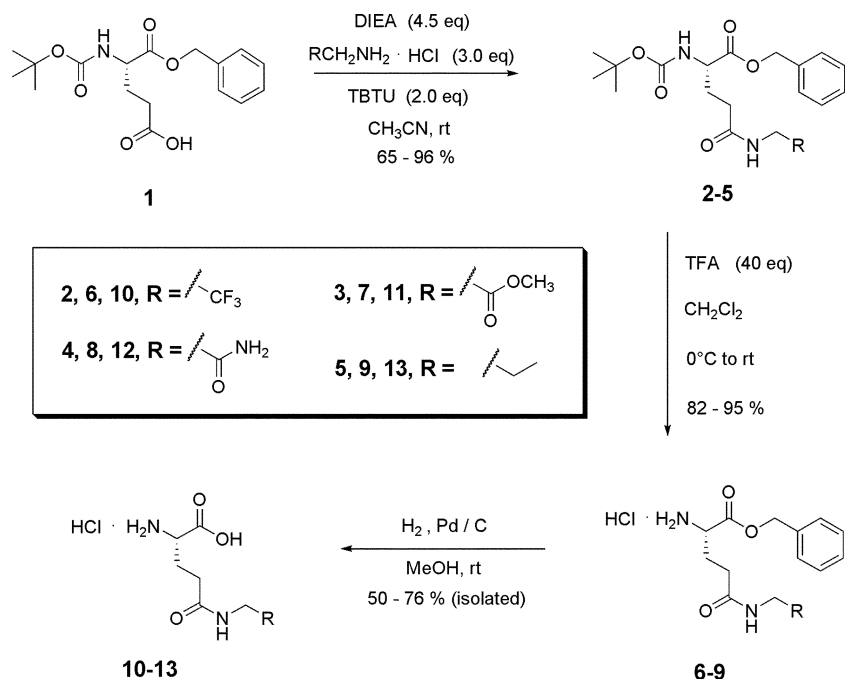
Results and discussion

Substrate analogue synthesis

All of the substrate analogues tested in the course of this study were prepared in excellent yields by standard peptide coupling methods. Most of the substrates were prepared according to Scheme 2, involving TBTU coupling of various amines with the γ-carboxylate group of the α-benzyl ester of *N*-Boc-L-glutamic acid. The details of this coupling are provided as the general method A. This coupling was followed by removal of the Boc group with TFA (general method B) and removal of the benzyl ester by hydrogenolysis (general method C). Two substrates
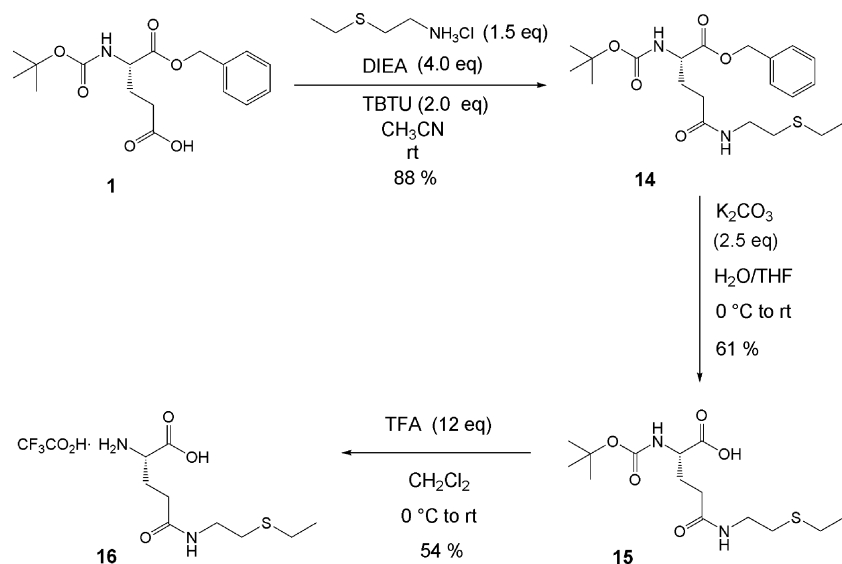
were prepared by slightly different synthetic routes. During the preparation of L-γ-glutamyl-2-(ethylthio)ethyl amide (**16**), benzyl ester hydrolysis preceded removal of the Boc group (Scheme 3). The synthesis of L-γ-GluAlaGly (**20**) was effected according to a simple peptide coupling with L-AlaGly according to a protocol previously developed in the group.[22] This synthesis, shown in Scheme 4, involves the *in situ* preparation of a *p*-nitrophenyl ester intermediate prior to peptide coupling, prior to hydrogenolysis of the benzyl ester and removal of the Boc protecting group.
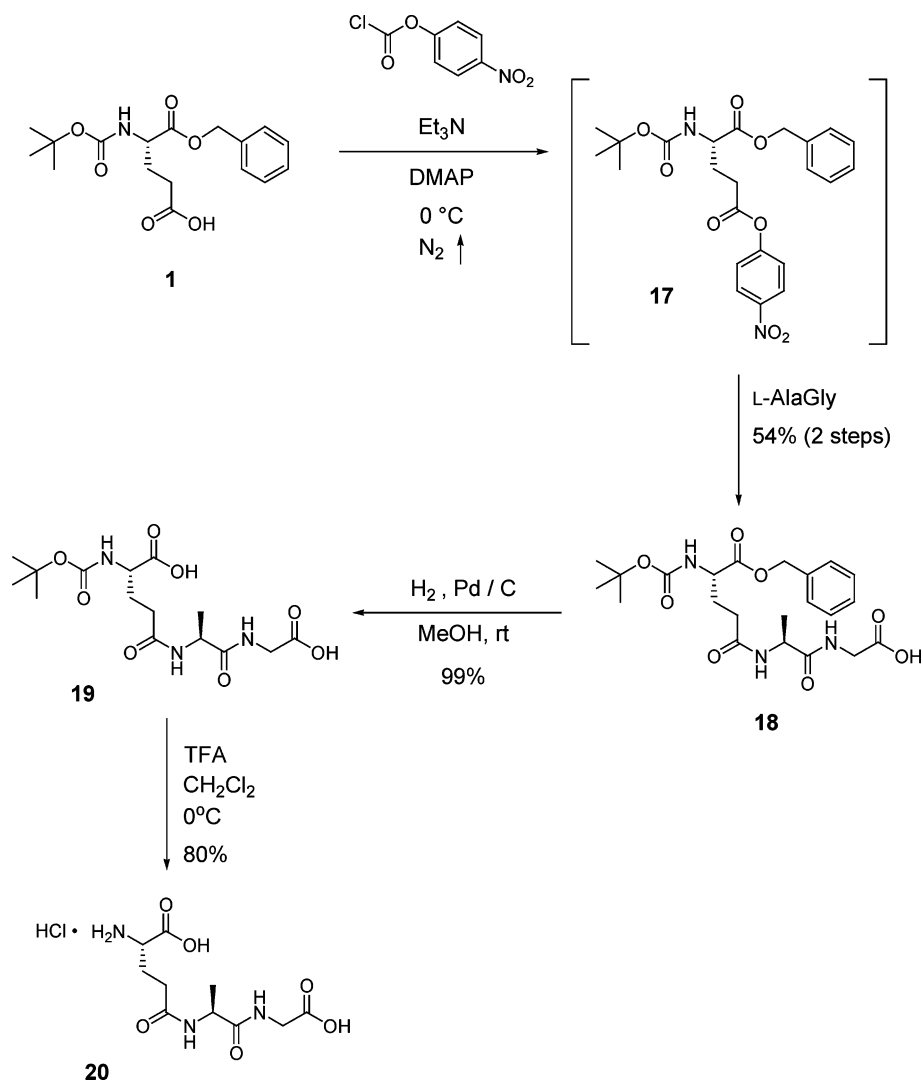
HPLC analysis

The amino acids and peptides found in the enzymatic reaction mixtures of these substrate analogues do not possess a strong chromophore, so a common derivatization method was used to permit their facile detection. Dabsyl chloride derivation of amino acids is well-known[23] and was optimized herein, with respect to the pH of the amino acid solution and the solvent of the dabsyl chloride stock solution used. Briefly, the optimal pH for the derivatization reaction was determined to be 9.0.[24,25] At lower

Scheme 2 Synthesis of the majority of γ-glutamyl amides studied herein.

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Scheme 3 Synthesis of L-γ-glutamyl-2-(ethylthio)ethyl amide.

Scheme 4 Synthesis of L-γ-GluAlaGly.

3792 | *Org. Biomol. Chem.*, 2006, **4**, 3790–3801

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pH, primary amine groups are predominantly protonated and slow to react, whereas at higher pH, background hydrolysis of dabsyl chloride becomes problematic. Concerning the co-solvent, although dabsyl derivatizations have been carried out previously in acetonitrile, we found that the greater solubility of dabsyl chloride in acetone allows for higher concentrations to be used, resulting in more rapid derivatization.

Subsequent separation of derivatized substrate analogues and enzyme reaction products was accomplished by reversed-phase HPLC. The elution gradients reported in the ESI† generally permitted baseline resolution of the amino acid and peptide derivatives. Making reference to an internal standard, the quantitative analysis of these reaction products from these HPLC chromatograms was found to be linear and accurate over the concentration range of 10–1000 μM in the enzymatic reaction mixture.[26] Although many amino acids were found to react rapidly and reproducibly with DABS-Cl, glutamine was chosen as an internal standard since it elutes from the HPLC column with a retention time similar to, but distinct from the other reaction components assayed in this application, providing a means for the reliable determination of its concentration, normalizing for variance in injection volume.[27]

In a typical application of this discontinuous assay to the kinetic study of GGT-mediated reactions, enzymatic reaction mixtures were prepared on such a scale as to provide several aliquots that were removed at different times throughout the initial rate time course. These aliquots were immediately quenched with TCA, stopping the enzymatic reaction through precipitation of the enzyme. Once a series of samples were accumulated, they were analysed by HPLC, typically overnight, using an autosampler. Admittedly, discontinuous assay methods are often tedious and less convenient for application to kinetic analysis; however, the availability of an autosampler allowed the injections and subsequent chromatographic separations to be completely automated, rendering this method almost as practical as a continuous reaction assay. In this way, kinetic data representing linear initial rates were obtained rapidly for a variety of substrate analogue L-γ-glutamyl amides (see ESI†).

pH–Rate profile for rate-limiting acylation of GGT by GSH

Kinetic studies were thus carried out at pH 8.0 and 37 °C in the presence of varied concentrations of L-γ-glutamyl donor substrates and a high concentration (20 mM, >6-fold K_M) of GlyGly, an excellent acceptor substrate for GGT-mediated transpeptidation.[9,13] This high concentration of GlyGly also assures that the deacylation step of the acyl-transfer mechanism is rapid and the preceding acylation step is rate-limiting and manifested as k_{cat}. Under these conditions, the slower hydrolysis reaction and the adventitious autotranspeptidation reaction of the γ-glutamyl substrate are apparently minimized.[1,10,15,18,19] Initial rates measured this way were plotted against the substrate concentration at which they were obtained, resulting in Michaelis–Menten plots of which a representative example is shown in Fig. 1.

The generality of the HPLC method also permitted the kinetic evaluation of GSH, the non-chromogenic native donor substrate of GGT. A pH–rate plot was constructed using GSH as donor substrate and GlyGly as acceptor substrate, according to the equation described in the Experimental section. The bell-shaped
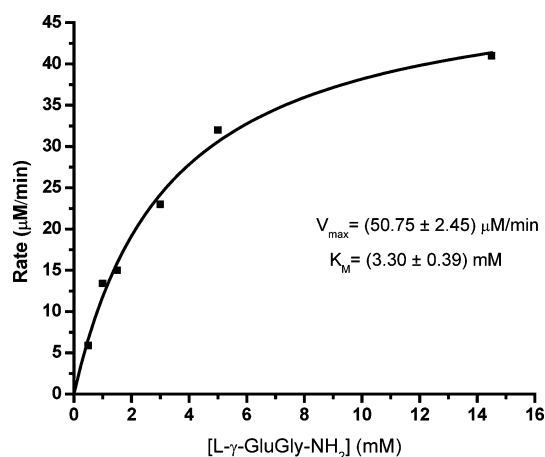
Fig. 1 Michaelis–Menten plot of the rate of GGT-mediated transpeptidation reaction between L-γ-glutamylglycinamide and 20 mM GlyGly at pH 8.0 and 37 °C. The standard deviations reported for the kinetic parameters derive from the non-linear regression for the hyperbolic line through data obtained from single kinetic runs.

curve thus obtained for GSH (Fig. 2) gives macroscopic kinetic pK_a values of 7.88 and 8.37, similar to those obtained previously by another method,[15] for ionizations of the E·S complex. This curve illustrates the dependence of the catalytic mechanism for acylation from the native donor substrate on the ionization states of (at least) two residues, one of which must be in its basic form and another that is active in its acidic form. The lower kinetic pK_a value has previously been attributed[9,28] to a histidine imidazole. Subsequent mutagenesis studies showed that replacement of the two histidine residues of the small subunit of human GGT dramatically reduced the efficiency of the mutant enzyme to 2% residual activity.[28] This is certainly suggestive of the involvement of these residues in a general acid–base mechanism[15,16] but does not exclude the possibility that other ionizable groups may be involved.[29] For example, other workers have suggested that the pH dependence of the acidic limb reflects the pK_a of GlyGly, the acceptor substrate
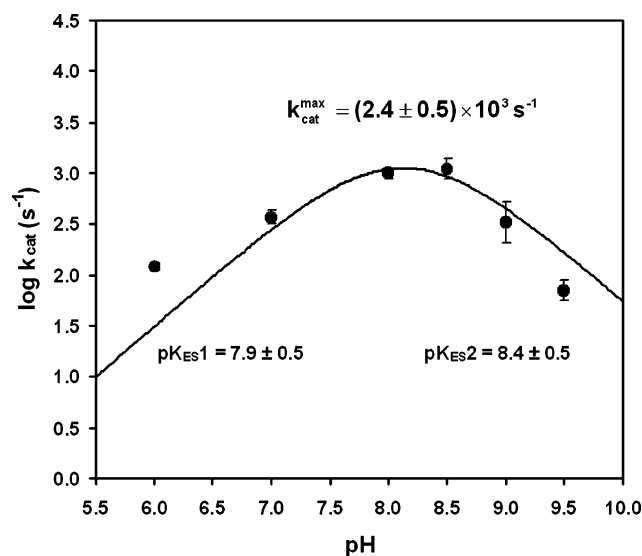

Fig. 2 pH–Rate profile for the GGT-mediated reaction of GSH with GlyGly at 37 °C.

common to these studies, suggesting that the deprotonated form of GlyGly (pK_a 8.13) is the true acceptor.[15,30] In either case, the present work confirms the kinetic pK_a values measured for the GGT-mediated reaction of GSH with GlyGly. The general HPLC method used herein could be applied to the study of a series of acceptor substrates to test this hypothesis concerning the lower kinetic pK_a value. For example, at a relatively high concentration of GSH (4 mM), the value of k_{cat} for L-AlaGly as acceptor substrate was determined to be 250 ± 30 s^{-1} ($K_M = 1.2 \pm 0.3$ mM). However, the work described herein focuses primarily on GGT acylation using donor substrate analogues.

Brønsted plots for substrate analogues

The flexibility of the method described herein permitted further investigation of the GGT-mediated transpeptidation reactions between GSH or a series of L-γ-glutamyl amide donor substrates and GlyGly as acceptor substrate (Table 1). Also shown in Table 1 are the pK_a values[31,32] for the conjugate acids of the amine leaving groups of the acylation reaction. It is apparent from Table 1 that the relative reactivities of the different donor substrates studied herein fall into three categories, their k_{cat} values for rate-limiting acylation separated roughly by an order of magnitude. The native tripeptide substrate, GSH, undergoes transpeptidation turnover most rapidly, while dipeptide substrates and finally simple γ-glutamyl alkyl amides are slower. This tendency, represented in Fig. 3, does not correlate simply with the nucleofugality of the leaving group during the acylation step. Reaction efficiency is apparently dominated by unidentified interactions related to gross substrate structure and affinity, rather than limited to the
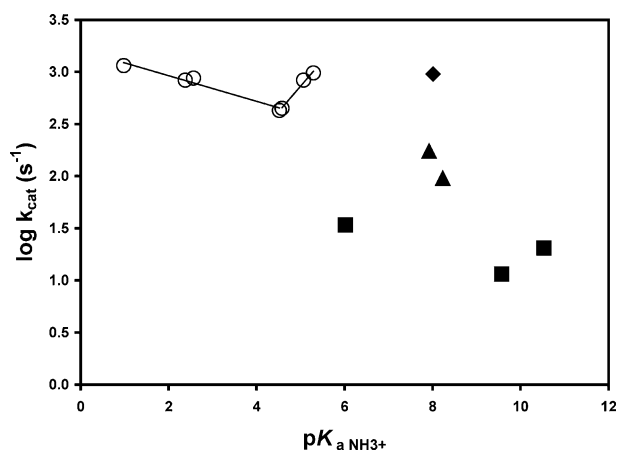
Fig. 3 Brønsted plot for the acylation of GGT by a series of L-γ-glutamyl substrates in the presence of 20 mM GlyGly at pH 8.0 and 37 °C. p$K_{a \, NH3+}$ refers to the pK_a of the conjugate acid ammonium of the amine leaving group. Data obtained previously for a series of anilides[15] are shown as circles. Data obtained in this work are shown in squares for a series of alkyl amides, triangles for dipeptides and as a diamond for GSH (k_{cat}, Table 1).

basicity of the departing amine. Simply stated, among the substrate analogues tested, the turnover of the acylation step appears to increase with the similarity of the leaving group to CysGly, the initial product of the reaction with the native substrate GSH.

Also shown in Fig. 3 are k_{cat} values previously obtained for acylation of GGT by a series of γ-glutamyl anilides.[15] These data were obtained under similar conditions, using a relatively high concentration of GlyGly as an acceptor substrate, allowing

Table 1 Kinetic parameters (k_{cat}, K_M) determined for the series of γ-glutamyl substrate analogues studied herein

Donor substrate	X	p$K_{a \, NH3+}$[a]	k_{cat}[b]/s^{-1}	K_M[c]/mM	k_{cat}/K_M/mM^{-1} s^{-1}
L-γ-Glutamyl-L-cysteinylglycine (glutathione, GSH)		8.01	990	0.67	1480
L-γ-Glutamylglycine methyl ester		7.92	172	2.78	61.9
L-γ-Glutamylglycinamide		8.23	95.9	3.30	29.1
L-γ-Glutamylpropylamide		10.53	20.2	0.71	28.5
L-γ-Glutamyl-2-(ethylthio)ethylamide		9.57	11.6	0.49	23.7
L-γ-Glutamyl-2,2,2-trifluoroethylamide		6.02	33.9	2.35	14.4

[a] From ref. 31 and 32. [b] Relative error for k_{cat} values was ≤6% (SD) from non-linear regression. [c] Relative error for K_M values was ≤15% (SD) from non-linear regression.

comparison with the data obtained herein. In general, two peculiarities of the anilide substrates are evident from this comparison—their greater reactivity than alkyl amides and dipeptides and the distinctive free-energy relationship with the basicity of their departing aniline groups. The enhanced reactivity of anilides compared to alkyl amides toward acyl transfer reactions is well-known, and they have long been studied as models for amide hydrolysis reactions for this reason.[33] It is therefore not surprising that the anilides studied previously are more efficient substrates than the alkyl amides and peptides studied herein. It is clear from Fig. 4 that the positive Brønsted correlation for the most basic anilines does not continue with the more basic amine leaving groups studied herein, for example. The non-linear free-energy relationship for the k_{cat} values measured previously for the anilides was ascribed to their concerted protonation and departure from the tetrahedral intermediate formed during the acylation step.[15] However, no obvious trend is apparent for the k_{cat} values measured for the substrate analogues studied herein.
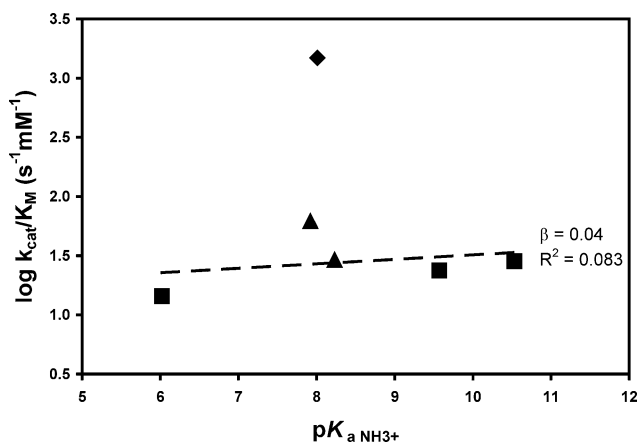
Fig. 4 Brønsted plot for the acylation of GGT by a series of L-γ-glutamyl substrates in the presence of 20 mM GlyGly at pH 8.0 and 37 °C. $pK_{a\,NH3+}$ refers to the pK_a of the conjugate acid ammonium of the amine leaving group. Data are shown in squares for a series of alkyl amides, triangles for dipeptides and as a diamond for GSH (k_{cat}/K_M, Table 1).

Rather, a more linear correlation is observed for the Brønsted plot constructed from the ratio of k_{cat}/K_M (Fig. 4), suggesting that the rate-limiting step is the reaction of the free enzyme with the substrate rather than the decomposition of a covalent tetrahedral intermediate, as postulated for the anilides. From this plot it is again apparent that the relative efficiencies of the different donor substrates studied herein fall into at least two categories, their specificity constants (k_{cat}/K_M) separated by roughly two orders of magnitude. The native tripeptide substrate, GSH, undergoes transpeptidation most efficiently, while the dipeptide and γ-glutamyl alkyl amide substrates are far less efficient. The near-zero slope obtained for the substrate analogues suggests there is very little development of charge on the nitrogen of the leaving group at the transition state of the rate-determining step. Taken together, the results shown in Fig. 3 and 4 are consistent with rate-limiting nucleophilic attack of GGT on substrate analogues, followed by rapid protonation and expulsion of the amine leaving group to form the acyl enzyme. Again, this mechanism differs markedly from that postulated previously[15] for the acylation of
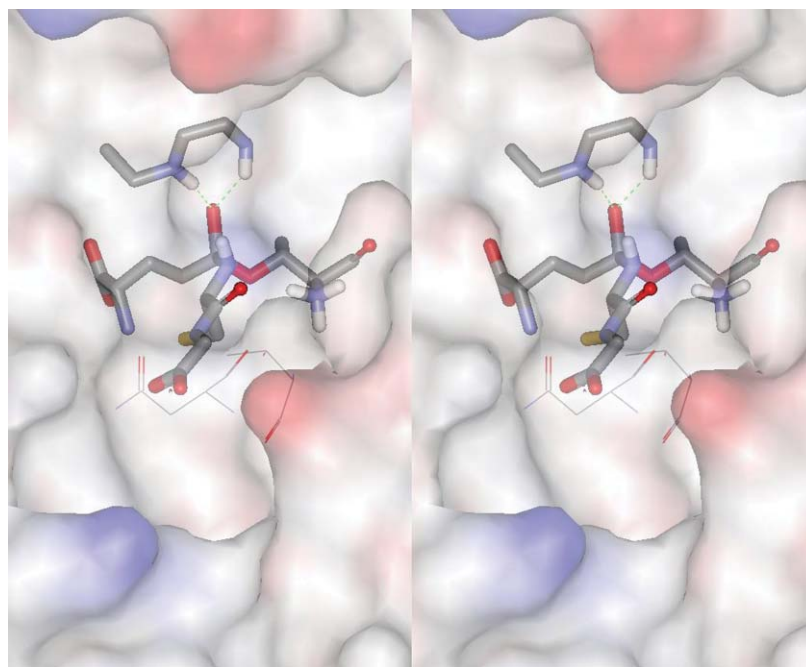
GGT from a series of activated anilides, where nucleophilic attack is apparently faster than the rate-limiting breakdown of the tetrahedral intermediate. Studies of non-enzymatic amide hydrolysis (or the microscopic reverse reaction) have shown that either formation or breakdown of the tetrahedral intermediate can be rate-limiting and that related Brønsted slopes can be very weakly negative.[34,35] Alternatively, similar studies[36,37] with chymotrypsin, a hydroxyl-nucleophile enzyme that catalyzes analogous acyl transfer reactions, have shown that weak Brønsted slopes may be observed when different means of substrate activation cancel each other. For example, decreasing electrophilicity within a series of substrates may be compensated by increasing hydrogen bonding in the oxyanion hole of chymotrypsin—or in this case, with the conserved glycine residues of GGT (Fig. 5).
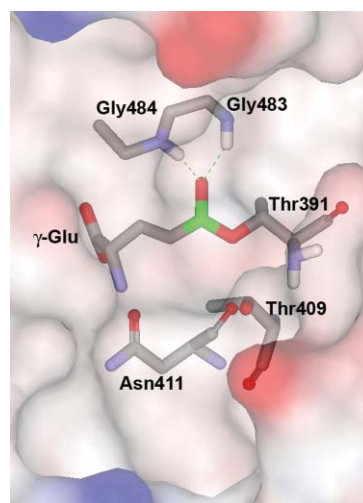
Fig. 5 Crystal structure of γ-glutamyl acyl-enzyme intermediate of *E. coli* GGT from ref. 21 (PDB entry 2DBW). Active site nucleophile Thr391, hydrogen bonding Gly382 and Gly383 and conserved binding site residues Thr409 and Asn411 are shown in stick form.

Structural considerations

The differences between the mechanisms proposed for acylation from anilides or from alkyl amides may be accounted for by the structural differences between the series of substrates studied and their accompanying interactions with the enzyme. Recently, Fukuyama and coworkers published the first high resolution GGT crystal structure for the *E. coli* enzyme.[21] In their publication, they draw attention to the exposed cavity beside the γ-glutamyl carbonyl of the crystalline acyl-enzyme intermediate (Fig. 5). When we used their crystal structure to construct the tetrahedral intermediate formed from nucleophilic attack on the native substrate GSH, the CysGly leaving group could be manually docked into the adjacent putative binding site, providing a compelling image of a potentially relevant mechanistic intermediate (Fig. 6). From this model it is apparent that the dimensions of the cavity are appropriate for binding CysGly and that the departing nitrogen is correctly positioned to be protonated by the ammonium group of the N-terminal nucleophilic Thr391 residue. The floor of the cavity is formed by residues such as Asn411 and Thr409 in the *E. coli* enzyme that are conserved in the rat and human

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Fig. 6 Stereo view of model of anionic GSH tetrahedral intermediate constructed from crystalline acyl-enzyme intermediate, intended to demonstrate the scale of the putative Cys–Gly subsite. Residues can be identified from Fig. 5, with Thr409 and Asn411 shown in line form and all labels removed for the sake of clarity. Image generated using Accelrys Discovery Studio Visualizer.

enzymes. Furthermore, in the model, Thr409 can form a close dipole interaction with the cysteine sulfhydryl while Asn411 can form a hydrogen bond with the glycine NH of the leaving group. The glycine carboxylate extends to the edge of the binding site where its potential interactions with solvent may also contribute to orienting the CysGly leaving group in the cavity. While these interactions will be verified through more rigorous modelling studies and/or future crystallographic efforts, the existence and nature of the cavity allows meaningful interpretation of the kinetic data presented herein.

The γ-glutamyl anilide substrates studied previously would appear to owe their reactivity to their predisposition to nucleophilic attack,[33] leading to acylation through rate-limiting breakdown of their tetrahedral intermediate[15] without assistance from any specific interaction between the enzyme and the aniline leaving group. Alkyl amides are less activated and subsequently less efficient substrates such that nucleophilic attack may be the rate-limiting step in their acylation of GGT. As the resemblance between these substrate analogues and the native substrate GSH increases, their efficiency increases also. This may be due to the ability of peptidic substrates to take advantage of specific interactions in the binding site identified by Fukuyama. Ultimately, the binding of CysGly in the site would realise optimal interactions and imparts to GSH its optimal reactivity. These interactions may help to distort the γ-glutamyl amide group, disrupting N–C=O resonance and rendering the carbonyl more susceptible to nucleophilic attack, a mechanism suggested to be important for amide cleavage by proteases as well.[38] This form of activation would be more important than remote inductive effects expected from electron withdrawing substituents on the amine leaving group, which may explain why the slope of Fig. 4 is near zero, rather than negative as expected for a series of substrates of varying electrophilicity.

Experimental

Materials

General information. Dabsyl chloride and synthetic starting materials were purchased from Sigma-Aldrich; dabsyl chloride was recrystallised from acetone.[39] All aqueous solutions were prepared from water purified and deionized by a Millipore Milli-Q Biocell system. All HPLC solvents including acetone were purchased from VWR. HPLC solvents were filtered by a Millipore filter (0.22 μm). Sample preparation was carried out using 0.22 μm filters and 2 mL micro tubes with caps purchased from Sarstedt and automated sample injection using 300 μL crimp vials with clear snap-close lids purchased from VWR. Separation and detection was effected using a Hitachi D-7000 HPLC, comprising a model L-7100 pump, a Higgins Targa C18 5 μM HPLC column (250 × 4.6 mm, Chromabec), an automatic injection system (model L-7200) and a photodiode detector (model L-7450).

Enzyme preparation. GGT was purified from rat kidney according to a modified protocol[15] based on the method published by Tate and Meister.[1,19]

Synthesis of glutathione analogue substrates.

Amide coupling reaction: general method A. Diisopropylethylamine (DIEA) (2.32 mL, 13.3 mmol, 4.5 eq) was added dropwise under an inert atmosphere at room temperature to a solution of *N*-Boc-L-glutamic acid α-benzyl ester **1** (1.0 g, 2.9 mmol, 1.0 eq) in 100 mL anhydrous acetonitrile. The solution was stirred for about ten minutes prior to the addition of alkyl amine in the form of its hydrochloride salt (8.9 mmol, 3.0 eq). After complete dissolution, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium

tetrafluoroborate (TBTU) (1.9 g, 5.9 mmol, 2.0 eq) was added and the reaction mixture was stirred prior to addition of 100 mL of ethyl acetate. The organic phase was washed with water (2 × 30 mL), 0.1 M HCl solution (20 mL), a solution of 5% NaHCO$_3$ (2 × 20 mL) and finally with saturated NaCl (20 mL). The organic phase was dried over MgSO$_4$, filtered and concentrated under reduced pressure. The alkyl amide product was obtained as an off-white solid.

N-Boc-L-γ-glutamyl-2,2,2-trifluoroethylamide α-benzyl ester (2). The general coupling procedure was followed using 2,2,2-trifluoroethylamine HCl (1.2 g, 8.9 mmol, 3.0 eq) and stirring for 6 hours. Amide **2** was obtained as a beige solid (1.18 g, 95%). R_f: 0.66 (70 : 30 EtOAc–hexane). Mp 96–97 °C. $[a]_D^{20}$: −2.3° (c = 1.00; CHCl$_3$). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.37–7.33 (m, 5 H), 5.17 (q, J = 10.2 Hz, 2 H), 4.17 (m, 1 H), 3.89–3.85 (m, 2 H), 2.35 (t, J = 7.3 Hz, 2 H), 2.15–2.12 (m, 1 H), 1.94–1.92 (m, 1 H), 1.43 (s, 9 H). ^{13}C-NMR (100 MHz, CDCl$_3$) δ (ppm): 172.3, 171.9, 155.9, 135.0, 128.5, 128.4, 128.2, 124.0 (q, J_{C-F} = 278.6 Hz), 80.2, 67.1, 52.8, 40.0 (q, J_{C-F} = 34.4 Hz), 38.4, 32.0, 28.0 HRMS C$_{19}$H$_{25}$F$_3$N$_2$O$_5$ (M + H)$^+$ calc: 419.1716, found: 419.1783.

N-Boc-L-γ-glutamylglycine methyl ester α-benzyl ester (3). The general coupling procedure was followed using glycine methyl ester HCl (1.1 g, 8.9 mmol, 3.0 eq) and stirring for 5 hours. Amide **3** was obtained as a yellowish white solid (0.98 g, 82%). R_f: 0.48 (70 : 30 EtOAc–hexane). Mp 88–90 °C. $[a]_D^{20}$: +2.3° (c = 1.00; CHCl$_3$). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.39–7.31 (m, 5 H), 5.17 (q, J = 10.3 Hz, 2 H), 4.17 (m, 1 H), 3.91 (s, 2 H), 3.71 (s, 3 H), 2.35 (t, J = 7.6 Hz, 2 H), 2.15–2.13 (m, 1 H), 1.92 (m, 1 H), 1.43 (s, 9 H). ^{13}C-NMR (100 MHz, CDCl$_3$) δ (ppm): 172.6, 170.8, 156.2, 135.7, 129.0, 128.9, 128.7, 80.5, 67.6, 53.3, 52.7, 41.7, 32.6, 29.4, 28.7. HRMS C$_{20}$H$_{28}$N$_2$O$_7$ (M + H)$^+$ calc: 409.1897, found: 409.1965.

N-Boc-L-γ-glutamylglycinamide α-benzyl ester (4). The general coupling procedure was followed using glycinamide HCl (0.33 g, 2.9 mmol, 2.0 eq) and stirring for 18 hours. Amide **4** was obtained as a beige solid (0.38 g, 65%). R_f: 0.83 (70 : 30 EtOAc–hexane). Mp 43–45 °C. $[a]_D^{20}$: +4.5° (c = 1.00; CHCl$_3$). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.37–7.31 (m, 5 H), 5.17 (q, J = 9.5 Hz, 2 H), 4.18–4.16 (m, 1 H), 3.81 (q, J = 9.3 Hz, 2 H), 2.37 (t, J = 7.4 Hz, 2 H), 2.16–2.13 (m, 1 H), 1.91 (m, 1 H), 1.43 (s, 9 H). ^{13}C-NMR (100 MHz, CDCl$_3$) δ (ppm): 173.0, 172.8, 172.4, 156.2, 136.6, 129.1, 128.9, 128.7, 128.5, 80.6, 67.7, 53.4, 43.3, 32.3, 28.7. HRMS C$_{19}$H$_{27}$N$_3$O$_6$ (M + H)$^+$ calc: 394.1900, found: 394.1991.

N-Boc-L-γ-glutamylpropylamide α-benzyl ester (5). The general coupling procedure was followed using propylamine HCl (0.85 g, 8.9 mmol, 3.0 eq) and stirring for 4 hours. Amide **5** was obtained as a white solid (1.08 g, 96%). R_f: 0.62 (70 : 30 EtOAc–hexane). Mp 87–88 °C. $[a]_D^{20}$: −3.5° (c = 1.00; CHCl$_3$). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.37–7.34 (m, 5 H), 5.17 (m, 2 H), 4.17 (m, 1 H), 3.11 (m, 2 H), 2.27 (m, 2 H), 2.17–2.13 (m, 1 H), 1.91 (m, 1 H), 1.45 (m, 2 H), 1.43 (s, 9 H), 0.92 (m, 3 H). ^{13}C-NMR (100 MHz, CDCl$_3$) δ (ppm): 172.0, 171.8, 155.7, 135.2, 128.4, 128.3, 128.2, 79.8, 67.0, 53.1, 41.2, 32.4, 28.6, 28.1, 22.5, 11.2. HRMS C$_{20}$H$_{30}$N$_2$O$_5$ (M + H)$^+$ calc: 379.2155, found: 379.2242.

Removal of Boc protecting group: general method B

To a solution of N-Boc-L-γ-glutamylalkylamide α-benzyl ester **2–5** (2.9 mmol, 1.0 eq) in 50 mL of anhydrous dichloromethane was added, dropwise at 0 °C, trifluoroacetic acid (TFA) (4.5 mL, 59 mmol, 20 eq). The solution was stirred at 0 °C for about 30 minutes and then at room temperature for around 2 hours. Then 4.5 mL of TFA (20 eq) at 0 °C was added again. The solution was stirred at 0 °C for another 30 minutes, and then again for 2 hours at room temperature. The solvent was then removed under reduced pressure and the crude product was washed with CH$_2$Cl$_2$ (2 × 10 mL) and with cyclohexane (4 × 10 mL). This TFA salt of the product amine was then dissolved in 0.1 M HCl (50 mL) and washed with diethyl ether (3 × 15 mL). The aqueous phase was evaporated under reduced pressure to give the product amine as a yellowish-white HCl salt.

L-γ-Glutamyl-2,2,2-trifluoroethylamide α-benzyl ester (6). The general procedure for Boc group removal was followed using 1.2 g of N-Boc-L-γ-glutamyl-2,2,2-trifluoroethylamide α-benzyl ester **2** to give amine **6** as a yellowish-white solid HCl salt (0.98 g, 94%). R_f: 0.13 (70 : 30 EtOAc–hexane). Mp 125–127 °C. $[a]_D^{20}$: +5.1° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.41–7.36 (m, 5 H), 5.28 (m, 2 H), 4.16–4.13 (m, 1 H), 3.89–3.84 (m, 2 H), 2.51–2.47 (m, 2 H), 2.21–2.17 (m, 2 H). ^{13}C-NMR (100 MHz, CD$_3$OD) δ (ppm): 174.3, 170.0, 136.3, 129.9, 129.6 (2 peaks), 127.2 (q, J_{C-F} = 278.2 Hz), 69.2, 53.5, 41.6 (q, J_{C-F} = 34.8 Hz), 31.8, 26.8. HRMS C$_{14}$H$_{17}$F$_3$N$_2$O$_3$ (M + H)$^+$ calc.: 319.1191, found: 319.1260.

L-γ-Glutamylglycine methyl ester α-benzyl ester (7). The general procedure for Boc group removal was followed, but without washing with ether or formation of the HCl salt, using N-Boc-L-γ-glutamylglycine methyl ester α-benzyl ester **3** (0.98 g, 2.4 mmoles, 1.0 eq) to give the TFA salt of amine **7** as a brownish oil (0.97 g, 95%). R_f: 0.08 (70 : 30 EtOAc–hexane). $[a]_D^{20}$: +3.6° (c = 1.00; H$_2$O). ^{1}H-NMR (300 MHz, CD$_3$OD) δ (ppm): 7.41–7.34 (m, 5 H), 5.29 (m, 2 H), 4.16 (m, 1 H), 3.91 (s, 2 H), 3.70 (s, 3 H), 2.49 (t, J = 7.0 Hz, 2 H), 2.25–2.10 (m, 2 H). ^{13}C-NMR (100 MHz, CD$_3$OD) δ (ppm): 174.5, 171.9, 170.1, 161.0 (q, J_{C-F} = 38.0 Hz), 136.3, 129.8, 129.7, 129.6, 117.2 (q, J_{C-F} = 287.9 Hz), 69.2, 53.6, 52.7, 41.9, 31.8, 27.0. LRMS C$_{15}$H$_{20}$N$_2$O$_5$ (M + H)$^+$ calc.: 309.1, found: 309.1.

L-γ-Glutamylglycinamide α-benzyl ester (8). The general procedure for Boc group removal was followed using N-Boc-L-γ-glutamylglycinamide α-benzyl ester **4** (0.33 g, 0.8 mmol, 1.0 eq) to give amine **8** as a yellowish-white solid HCl salt (0.28 g, 82%). R_f: 0.05 (70 : 30 EtOAc–hexane). Mp 57–60 °C. $[a]_D^{20}$: +5.8° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.44–7.36 (m, 5 H), 5.30 (m, 2 H), 4.17 (t, J = 6.3 Hz, 1 H), 3.85 (m, 2 H), 2.50 (m, 2 H), 2.25 (m, 1 H), 2.18–2.14 (m, 1 H). ^{13}C-NMR (100 MHz, CD$_3$OD) δ (ppm): 174.5, 174.4, 170.2, 136.4, 129.9 (2 peaks), 129.8, 69.3, 53.7, 43.0, 32.0, 26.9. LRMS C$_{14}$H$_{19}$N$_3$O$_4$ (M + H)$^+$ calc.: 294.1, found: 294.2.

L-γ-Glutamylpropylamide α-benzyl ester (9). The general procedure for Boc group removal was followed using N-Boc-L-γ-glutamylpropylamide α-benzyl ester **6** (1.08 g, 2.9 mmol, 1.0 eq) giving **9** as a yellowish oil (1.01 g, 95%). R_f: 0.2 (70 : 30 EtOAc–hexane). $[a]_D^{20}$: +4.9° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, CD$_3$OD) δ (ppm): 7.44–7.35 (m, 5 H), 5.30 (m, 2 H), 4.15

(t, J = 6.4 Hz, 1 H), 3.11 (t, J = 7.3 Hz, 2 H), 2.42 (t, J = 6.9 Hz, 2 H), 2.19–2.13 (m, 2 H), 1.53–1.47 (m, 2 H), 0.91 (t, J = 7.5 Hz, 3 H). ^{13}C-NMR (100 MHz, CD$_3$OD) δ (ppm): 173.8, 170.1, 136.4, 129.9 (3 peaks), 69.2, 53.7, 42.3, 32.3, 27.2, 23.5, 11.7. HRMS C$_{15}$H$_{22}$N$_2$O$_3$ (M + H)$^+$ calc.: 279.1630, found: 279.1713.

Removal of benzyl protecting group: general method C

A solution of L-γ-glutamylalkylamide α-benzyl ester **6–9** (2.5 mmol, 1.0 eq) in 50 mL methanol was added, under inert atmosphere at room temperature, to palladium over carbon (10 wt%). The reaction mixture was allowed to stir for 18 hours under 150 psi hydrogen in a hydrogenation bomb. Once the reaction was complete, the reaction mixture was filtered over Celite® and washed with methanol. The methanol was then removed under reduced pressure and the crude reaction product was obtained as an oil. The product was then purified by precipitation from a methanol–ether mixture to give the final product as a white solid.

L-γ-Glutamyl-2,2,2-trifluoroethylamide (10). The general method C for removal of the benzyl group was followed using L-γ-glutamyl-2,2,2-trifluoroethylamide α-benzyl ester **7** (0.88 g, 2.5 mmol, 1.0 eq) and 0.09 g of palladium. The crude reaction product was obtained as an oil (0.6 g, 92%) and the purified product **10** was obtained as a white solid (0.39 g, 60%). Mp 167–170 °C (dec.). $[a]_D^{20}$: +14.1° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, D$_2$O) δ (ppm): 3.84–3.79 (m, 3 H), 2.45–2.40 (m, 2 H), 2.08 (m, 2 H). ^{13}C-NMR (100 MHz, D$_2$O) δ (ppm): 175.7, 172.3, 124.9 (q, J_{C-F} = 278.0 Hz), 53.0, 41.0 (q, J_{C-F} = 34.6 Hz), 31.7, 26.1. HRMS C$_7$H$_{11}$F$_3$N$_2$O$_3$ (M + H)$^+$ calc.: 229.0722, found: 229.0810.

L-γ-Glutamylglycine methyl ester (11). The general method C for removal of the benzyl group was followed using L-γ-glutamylglycine methyl ester α-benzyl ester **7** (0.97 g, 2.3 mmol, 1.0 eq). The crude reaction product, obtained as a yellow oil (0.71 g, 92%) was further purified by precipitation from methanol–ether to give final product **11** as a white solid (0.42 g, 55%). Mp 195 °C (dec.). $[a]_D^{20}$: +6.7° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, D$_2$O) δ (ppm): 4.04 (s, 2 H), 3.81 (t, J = 6.1 Hz, 1 H), 3.77 (s, 3 H), 2.52 (t, J = 7.5 Hz, 2 H), 2.20–2.16 (m, 2 H). ^{13}C-NMR (100 MHz, D$_2$O) δ (ppm): 176.1, 174.6, 173.1, 54.8, 53.6, 42.0, 31.9, 27.0. HRMS C$_8$H$_{14}$N$_2$O$_5$ (M + H)$^+$ calc.: 219.1, found: 219.1.

L-γ-Glutamylglycinamide (12). The general method C for removal of the benzyl group was followed using L-γ-glutamylglycinamide α-benzyl ester **8** (0.23 g, 0.7 mmol, 1.0 eq). The crude reaction product, obtained as a yellow oil (0.16 g, 95%) was further purified by precipitation from methanol–ether to give final product **12** as a white solid (0.13 g, 76%). Mp 190 °C (dec.). $[a]_D^{20}$: +11.3° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, D$_2$O) δ (ppm): 3.88 (t, J = 6.2 Hz, 1 H), 3.81 (s, 2 H), 2.45 (m, 2 H), 2.11–2.08 (m, 2 H). ^{13}C-NMR (100 MHz, D$_2$O) δ (ppm): 175.8, 175.1, 172.5, 53.3, 42.9, 31.6, 26.2. HRMS C$_7$H$_{13}$N$_3$O$_4$ (M + H)$^+$ calc.: 204.0906, found: 204.0979.

L-γ-Glutamylpropylamide (13). The general method C for removal of the benzyl group was followed using L-γ-glutamylpropylamide α-benzyl ester **9** (0.94 g, 3.0 mmol, 1.0 eq). The crude reaction product, obtained as a colourless oil (0.6 g, 89%) was further purified by precipitation from methanol–ether

to give final product **13** as a white solid (0.33 g, 50%). Mp 86–89 °C. $[a]_D^{20}$: +14.3° (c = 1.00; H$_2$O). ^{1}H-NMR (400 MHz, D$_2$O) δ (ppm): 4.05 (t, J = 6.1 Hz, 1 H), 3.14 (t, J = 6.9 Hz, 2 H), 2.50–2.45 (m, 2 H), 2.23–2.18 (m, 2 H), 1.53–1.48 (m, 2 H), 0.88 (t, J = 7.3 Hz, 3 H). ^{13}C-NMR (100 MHz, D$_2$O) δ (ppm): 174.8, 172.7, 53.3, 42.1, 32.1, 26.7, 22.5, 11.4. HRMS C$_8$H$_{16}$N$_2$O$_3$ (M + H)$^+$ calc.: 189.1161, found: 189.1243.

N-Boc-L-γ-glutamyl-2-(ethylthio)ethylamide α-benzyl ester (14). N-Boc-L-glutamic acid α-benzyl ester **1** (0.6 g, 1.78 mmol, 1.0 eq) was coupled with 2-(ethylthio)ethyl amine according to Method A, with the following exceptions: 1.5 eq of alkyl amine and 4.0 eq of DIEA were used (instead of 3.0 eq and 4.5 eq, respectively) and the reaction with TBTU was allowed to proceed overnight. Amide **14** was obtained as a beige solid (0.67 g, 88%). R$_f$: 0.23 (70 : 30 EtOAc–hexane). Mp 62–65 °C. $[a]_D$: −1.5 (c = 0.01; CHCl$_3$). ^{1}H-NMR (300 MHz, CDCl$_3$) δ (ppm): 7.33 (m, 5 H), 5.13 (q, J = 12.9 Hz, 2 H), 4.31 (m, 1 H), 3.38 (q, J = 6.3 Hz, 2 H), 2.62 (t, J = 6.6 Hz 2 H), 2.50 (q, J = 7.4 Hz, 2 H), 2.26–2.21 (m, 2 H), 2.17–2.14 (m, 1 H), 1.95–2.02 (m, 1 H), 1.41 (s, 9 H), 1.23 (t, J = 7.4, 3 H). ^{13}C-NMR (75 MHz, CDCl$_3$) δ (ppm): 172.6, 172.4, 156.2, 135.7, 128.9, 80.4, 78.0, 67.5, 60.8, 53.6, 39.0, 32.9, 31.5, 30.0, 28.7, 25.9, 15.1. HRMS C$_{21}$H$_{32}$N$_2$O$_5$S (M + H)$^+$ calc.: 424.5560, found: 424.2032.

N-Boc-L-γ-glutamyl-2-(ethylthio)ethylamide (15). A solution of N-Boc-L-γ-glutamyl-2-(ethylthio)ethylamide α-benzyl ester **14** (225 mg, 0.141 mmol, 1.0 eq) dissolved in a 1 : 1 mixture of H$_2$O–THF was cooled to 0 °C, followed by the addition of K$_2$CO$_3$ (219 mg, 1.58 mmol, 3 eq) to the reaction mixture. The mixture was stirred initially at 0 °C and allowed to warm to room temperature overnight. Water was then added (5 mL) and the reaction was stirred for another 5 hours. The THF was then removed under reduced pressure and the aqueous phase was acidified with 1 M HCl and extracted with 25 mL EtOAc. The organic phase was then dried over MgSO$_4$, filtered and removed under reduced pressure. The residue was dissolved in EtOAc and purified by flash chromatography (80 : 15 : 5 EtOAc–hexane–HOAc). Acid **15** was thus obtained as a colourless gum (107 mg, 61%). R$_f$: 0.55 (80 : 15 : 5 EtOAc–hexane–HOAc). $[a]_D$: +8.3 (c = 0.0029; CHCl$_3$). ^{1}H-NMR (300 MHz, CDCl$_3$) δ (ppm): 4.30 (m, 1 H), 3.48 (q, J = 6.1 Hz, 2 H), 2.70 (t, J = 6.6 Hz, 2 H), 2.57 (q, J = 7.4 Hz, 2 H), 2.44 (m, 1 H), 2.22–2.19 (m, 1 H), 2.17–2.04 (m, 1 H), 1.46 (s, 9 H), 1.28 (t, J = 7.4, 3 H). ^{13}C-NMR (75 MHz, CDCl$_3$) δ (ppm): 174.6, 174.0, 156.5, 80.7, 53.3, 39.2, 33.0, 30.6, 29.4, 28.8, 25.9, 15.2. HRMS C$_{14}$H$_{26}$N$_2$O$_5$S (M + H)$^+$ calc.: 334.4354, found: 334.1562.

L-γ-Glutamyl-2-(ethylthio)ethylamide (16). To a solution of N-Boc-L-γ-glutamyl-2-(ethylthio)ethylamide **15** (38 mg, 0.11 mmol, 1.0 eq) dissolved in dichloromethane (10 mL), TFA (0.026 mL, 0.342 mmol, 3 eq) was added dropwise at 0 °C. After stirring for 20 minutes, the reaction mixture was allowed to warm to room temperature. After stirring for another 90 minutes, another 3 eq aliquot of TFA was added dropwise at 0 °C over 20 minutes. The mixture was stirred for another 3.5 hours at room temperature and another 3 eq aliquot of TFA was added dropwise at 0 °C. The solution was allowed to warm to room temperature with stirring overnight. Solvent was then removed under reduced pressure. Cyclohexane was added and subsequently removed under reduced

3798 | *Org. Biomol. Chem.*, 2006, **4**, 3790–3801

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pressure 4 times. The residue was dissolved in CH_2Cl_2 and purified by elution from a 3 cm plug of silica gel, through rapid washing with 10 mL aliquots of hexane, EtOAc, CH_2Cl_2 and a subsequent gradient of MeOH in CH_2Cl_2. A white solid was thus obtained (23 mg, 54%). R_f: 0.56 (80 : 15 : 5 EtOAc–hexane–HOAc). Mp 198–201 °C. ^{1}H-NMR (300 MHz, D_2O) δ (ppm): 3.74 (t, J = 6.1 Hz, 1 H), 3.39 (t, J = 6.7 Hz, 2 H), 2.69 (t, J = 6.6 Hz, 2 H), 2.56 (q, J = 7.4 Hz, 2 H), 2.42–2.37 (m, 2 H), 2.14–2.09 (m, 2 H), 1.20 (t, J = 7.4 Hz, 3 H). ^{13}C-NMR (75 MHz, D_2O) δ (ppm): 175.0, 174.3, 54.5, 38.9, 31.8, 30.2, 26.8, 25.3, 14.2. HRMS $C_9H_{18}N_2O_3S$ (M + H)$^+$ calc.: 234.3222, found: 234.1038.

N-Boc-L-γ-glutamyl-AlaGly α-benzyl ester (18). N-Boc-L-glutamic acid α-benzyl ester **1** (100 mg, 0.296 mmol, 1.0 eq) was dissolved in 4 mL anhydrous acetonitrile under an inert atmosphere at 0 °C. Triethylamine (0.041 mL, 0.296 mmol, 1.0 eq) was then added dropwise, followed by p-nitrophenyl chloroformate (63 mg, 0.311 mmol, 1.05 eq). After stirring for 5 minutes, 4-dimethylaminopyridine (DMAP) (4 mg, 0.030 mmol, 0.1 eq) was added. After 45 minutes, excess p-nitrophenyl chloroformate was hydrolysed upon addition of 1 mL of a solution of triethylamine (0.164 mL, 1.184 mmol, 4.0 eq) in 4 mL water. After ∼10 minutes, L-AlaGly (173 mg, 1.184 mmol, 4.0 eq) was added to the remaining 3 mL of the aqueous triethylamine solution, and the resulting solution was added dropwise to the reaction mixture. The reaction was allowed to stir for 2.5 hours. Solvents were then removed under reduced pressure and the resulting residue was purified by flash chromatography (85 : 10 : 5 EtOAc–MeOH–HOAc). Tripeptide **18** was obtained as a colourless gum (66 mg, 54%). R_f: 0.48 (85 : 10 : 5 EtOAc–MeOH–HOAc). $[a]_D$: −4.2 (c = 0.005; CHCl$_3$). ^{1}H-NMR (300 MHz, CD$_3$OD) δ (ppm): 7.30–7.36 (m, 5 H), 5.15 (q, J = 12.5 Hz, 2 H), 4.38 (m, 1 H), 4.17 (m, 1 H), 3.84 (s, 2 H), 2.34 (m, 2 H), 1.42 (s, 9 H), 2.15–2.12 (m, 1 H), 1.94–1.89 (m, 1 H), 1.34 (d, J = 7.1 Hz, 3 H). ^{13}C-NMR (75 MHz, CD$_3$OD) δ (ppm): 174.3, 173.5, 172.9, 157.1, 136.2, 128.6, 128.2, 79.7, 66.9, 53.7, 49.3, 31.8, 27.7, 27.1, 17.0. HRMS $C_{22}H_{31}N_3O_8$ (M + H)$^+$ calc.: 465.4949, found 465.2111.

N-Boc-L-γ-glutamyl-L-alanylglycine (19). N-Boc-L-γ-glutamyl-L-alanylglycine α-benzyl ester **18** (63 mg, 0.151 mmol) was dissolved in ∼5 mL of methanol. Palladium on charcoal (6 mg, 10 wt%) was then added to the reaction mixture, that was allowed to react overnight under 150 psi hydrogen in a hydrogenation bomb. The reaction mixture was then filtered over Celite® and washed with methanol. The solvent was then evaporated under reduced pressure to give **19** as a pale white gum (49 mg, 99%). R_f: 0.21 (85 : 10 : 5 EtOAc–MeOH–HOAc). $[a]_D$: −6.4 (c = 0.002; MeOH). ^{1}H-NMR (300 MHz, CD$_3$OD) δ (ppm): 4.38 (q, J = 7.1 Hz, 1 H), 4.08 (m, 1 H), 3.87 (s, 2 H), 2.36 (m, 2 H), 2.12 (m, 1 H), 1.95–1.89 (m, 1 H), 1.44 (s, 9 H), 1.36 (d, J = 7.1 Hz, 3 H). ^{13}C-NMR (75 MHz, CD$_3$OD) δ (ppm): 175.6, 174.4, 173.9, 172.9, 157.0, 79.5, 53.9, 49.4, 41.5, 32.0, 27.8, 27.8, 17.0. HRMS $C_{15}H_{25}N_3O_8$ (M + H)$^+$ calc.: 375.3775, found: 375.1642.

L-γ-Glutamyl-L-alanylglycine (20). To a solution of N-Boc-L-γ-glutamyl-L-alanylglycine **19** (41 mg, 0.125 mmol, 1.0 eq) dissolved in 15 mL CH_2Cl_2, TFA (0.127 mL, 0.752 mmol, 6.0 eq) was added dropwise over 20 minutes, with stirring, at 0 °C. The reaction mixture was then allowed to stir for 5 hours at room temperature. Solvent was removed under reduced pressure.

Cyclohexane was then added and subsequently removed under reduced pressure 4 times. The resulting residue was then dissolved in 25 mL EtOAc and extracted using ∼0.1 M aqueous HCl (3 × 25 mL). The aqueous phase was then evaporated under reduced pressure, giving the HCl salt of tripeptide **20** as a pale yellow gum (31 mg, 80%). R_f: 0.07 (80 : 15 : 5 EtOAc–hexane–HOAc) $[a]_D$: +18.4 (c = 0.01; CHCl$_3$). ^{1}H-NMR (300 MHz, D_2O) δ (ppm): 4.27 (q, J = 8.0 Hz, 1 H), 3.96 (t, J = 7.1 Hz, 1 H), 3.89 (s, 2 H), 2.31 (t, J = 7.0 Hz, 2 H), 1.99–1.93 (m, 2 H), 1.05 (d, J = 7.9 Hz, 3 H). ^{13}C-NMR (75 MHz, D_2O) δ (ppm): 176.1, 174.5, 173.4, 52.7, 50.0, 41.3, 31.1, 25.7, 16.9, 16.3. HRMS $C_{10}H_{17}N_3O_6$ (M + H)$^+$ calc.: 275.2643, found: 275.1117.

Methods

Assay procedure. Enzyme activity was normalized[15] using the standard GGT specific activity assay. The progress of the enzymatic reactions of the L-γ-glutamyl amide substrate analogues studied was followed by the formation of the transpeptidation product, L-γ-GluGlyGly. A test tube was charged with donor substrate giving final concentrations of 0.05–3.5 mM, after addition of 0.50 mL of a solution of 0.1 M glycylglycine, pH 8.0 (final concentration 20 mM) and dilution to 2.5 mL with 0.1 M HEPES buffer (pH 8.0). (HEPES was chosen as an appropriate reaction buffer since it does not contain a primary amine group for adventitious reaction with DABS-Cl during the subsequent derivatization step.) The solution was pre-incubated for 10 min at 37 °C and the reaction was initiated by the addition of 0.05–1.19 units of GGT, depending on the substrate analogue. At different times between 0 and 75 minutes, an aliquot of 195 μL was taken from the reaction vessel and transferred to a 2 mL microcentrifuge tube containing 60 μL of 40% trichloroacetic acid (TCA), thus quenching the reaction.[17] The enzyme precipitate was removed by centrifugation at 10 000 rpm (10 600 g) for 5 min at 4 °C. The supernatant fraction was then removed and placed in a second 2 mL microcentrifuge tube. A 20 μL aliquot of the standard 50 mM glutamine solution (in 0.2 M sodium bicarbonate, pH 9.0) was then added to each quenched reaction solution as an internal standard (0.5 mM final concentration). The concentration of derivatized reaction product in each quenched sample was determined by HPLC (see below) and plotted against reaction time to give linear initial rates. These acylation rates (measured under conditions where deacylation is rapid) were used to determine the kinetic parameters V_{max} and K_M according to the standard hyperbolic Michaelis–Menten equation by non-linear regression. The value for V_{max} was converted to k_{cat} by dividing by the concentration of enzyme used. The kinetic parameters of L-AlaGly as acceptor substrate were determined according to the same general procedure, using final concentrations of L-AlaGly between 0.2 and 5 mM and 4 mM of GSH as donor substrate.

Dabsyl chloride derivatization. Dabsyl derivatives were prepared according to a modified[26] procedure based primarily on the method published previously by Chang et $al.$[39] Firstly, the pH of each aliquot was adjusted to 9.0 ± 0.1 with NaOH. Next, an equal volume of a freshly prepared 20 mM solution of DABS-Cl in acetone was added to the solution. The resulting 2-fold molar excess of DABS-Cl is sufficient to ensure complete derivatization.[40] The mixture was then heated at 70 °C for 10–15 min, to ensure complete solubility, in 2 mL tubes equipped with airtight caps

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Org. Biomol. Chem., 2006, **4**, 3790–3801 | 3799

to prevent the evaporation of acetone. Under these conditions, derivatization is rapid and complete after heating to the point of solubilization. Finally, a 1 : 1 (v/v) solution of 40 mM sodium phosphate (pH 6.5)–ethanol was added, to a final volume of 2 mL, to dilute the derivatized sample prior to HPLC analysis. Derivatized samples were found to be stable at room temperature for several months[41] and can be stored at $-20\,°C$ for one year.[39]

HPLC analysis. Mixtures of dabsyl derivatives were separated and analysed by HPLC according to a modified[26] method based on a previously published procedure.[42] Samples were prepared as indicated above and analysed on a reversed-phase column, using an injection volume of 40 μL. The mobile phase for separation of dabsyl derivatives was comprised of two solvents: 25 mM monobasic potassium phosphate (pH 6.8) (solvent A) and an 80 : 20 mixture of acetonitrile–propan-2-ol (solvent B). The chromatographic profile used to separate products of the enzymatic reactions is shown in the ESI†. Note that for the enzymatic reactions of L-γ-glutamyl-2,2,2-trifluoroethylamide and L-γ-glutamylglycinamide, additional washing and column reconditioning steps were added to the profile, also reported in the ESI. In each case, the flow rate was 1 mL min^{-1} and the elution was carried out at ambient temperature.

Standard calibration plots. Authentic transpeptidation products were dissolved in sodium bicarbonate 0.2 M buffer (pH 9.0) according to the desired concentration. To a 0.5 mL aliquot of any given compound were added 20 μL of a standard 0.5 mM glutamine solution and 0.52 mL of a freshly prepared acetone solution of DABS-Cl, resulting in a final ≥2-fold excess of DABS-Cl, sufficient to ensure complete derivatization according to the method described above.[40] The derivatized sample was then diluted to a final volume of 2 mL with a solution of 1 : 1 40 mM sodium phosphate (pH 6.5)–ethanol prior to injection onto the HPLC. Subsequent quantification was accomplished by comparing the areas of the elution peaks of the analyte and the internal standard glutamine, measured by their absorbance at 420 nm. These curves displayed excellent linearity (see ESI†). The plot of the ratio of the area of the elution peak of L-γ-GluGlyGly to that of L-Gln, the internal standard, *versus* the injected concentration of L-γ-GluGlyGly, had a slope of 1.69×10^{-3} μM^{-1} ($R^2 = 0.994$). This effective extinction coefficient was used subsequently to determine the concentration of product L-γ-GluGlyGly in the quenched reaction aliquots. The quantitative measurement of L-γ-GluAlaGly as a transpeptidation product was accomplished similarly using peak heights, giving a slope of 0.853×10^{-3} μM^{-1} ($R^2 = 0.999$).

pH–Rate curve. The protocol described above for enzyme kinetic analysis was followed using 0.15–3.0 mM GSH and 20 mM GlyGly in 100 mM of the following buffers:[16] MES (pH 6.0), MOPS (pH 7.0), HEPES (pH 8.0) and CHES (pH 8.5, pH 9.0, pH 9.5). Kinetic parameters were determined from non-linear regression to the Michaelis–Menten equation as described above, and normalized k_{cat} values were subsequently determined from non-linear regression according to the following equation for two ionizations of the Michaelis ES complex:

$$k_{cat} = \frac{k_{cat}^{max}}{\frac{[H^+]}{K_{ES1}} + 1 + \frac{K_{ES2}}{[H^+]}}$$

Conclusions

In summary, we report a complementary method for kinetic analysis of the GGT-mediated transpeptidation reactions of non-chromogenic substrates, based on dabsyl derivatization of all primary amine products followed by separation and quantification by reversed-phase HPLC. This method is economical, simple, reproducible, sensitive and precise. Furthermore, its flexibility offers the potential for the analysis of enzymatic reactions of series of substrates that do not contain a chromophore, in particular those of the transamidases (EC 2.3.-.-) and peptidases (EC 3.4.-.-), whose peptidic substrates and products are not easily visualized quantitatively.

Using this kinetic technique, we obtained structure–function data for GGT-mediated transpeptidation that are not easily accessible by other methods. Kinetic data obtained for the acylation of GGT with the native substrate GSH as well as a series of dipeptide substrate analogues (with GlyGly as an efficient acceptor substrate) indicate that their reactivity is not dependent solely on the nucleofugality of the amine leaving group. The remarkable efficiency of GSH as a donor substrate, taken together with the higher turnover numbers obtained for dipetide substrate analogues, seems to suggest there are important enzyme–substrate affinity interactions that contribute more predominantly to acylation efficiency. Preliminary homology modelling based on the recently published[21] crystal structure of the similar *E. coli* enzyme suggests there may be specific interactions between the Cys–Gly leaving group of GSH and an adjacent binding sub-site that may contribute to the activation of GSH as an acyl-donor substrate. Further studies using peptidic substrate analogues that closely resemble the native substrates of GGT will be required to explore this hypothesis.

Abbreviations used

CHES, 2-(cyclohexylamino)ethanesulfonic acid; DABS, dabsyl, 4-dimethylaminoazobenzene-4′-sulfonyl; DIEA, diisopropylethylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholinoethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; TBTU, *N,N,N′,N′*-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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3800 | *Org. Biomol. Chem.*, 2006, **4**, 3790–3801

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