Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1561–1564

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

N-Me-*p*AB-Glu-γ-Glu-γ-Tyr(3-NO_2): An Internally Quenched Fluorogenic γ-Glutamyl Hydrolase Substrate

Jessica J. Pankuch and James K. Coward*

Departments of Chemistry and Medicinal Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA

Received 18 December 2000; accepted 15 January 2001

Abstract—A γ-glutamyl tripeptide containing an internally quenched fluorophore has been synthesized and shown to be a substrate for recombinant rat γ-glutamyl hydrolase. HPLC, LC–MS, and fluorescence spectra support the conclusion that selective hydrolysis occurs at the penultimate peptide bond. Preliminary data indicate that hydrolysis of this substrate can be monitored continuously to yield steady-state kinetic data

γ-Glutamyl hydrolase (GH, EC 3.4.19.9) catalyzes the hydrolysis of folylpoly-γ-glutamates, the intracellular forms of folate cofactors involved in one-carbon metabolism and thus provides one means of regulating cellular levels of folates.[1] Rat and human forms of GH have been cloned and overexpressed. Site-directed mutagenesis and calculations revealing high structural homology with the glutaminase domain of carbamoyl phosphate synthetase indicate that GH is a cysteine protease, in contrast to the zinc metalloprotease, glutamate carboxypeptidase II (GCPII, 'conjugase,' EC 3.4.17.21), which cleaves dietary folylpoly-γ-glutamates.[2]

We have developed fluoroglutamate-containing γ-glutamyl peptides as mechanistic probes for GH (Table 1). In earlier work investigating the effect of electron-deficient peptide bonds on the rate of GH-catalyzed hydrolysis, we observed that a methotrexate (4-amino-4-deoxy-10-methylpteroyl glutamate, AMPteGlu) derivative AMPte(2*RS*,4*RS*)-4-fluoroglutamyl-γ-glutamate was hydrolyzed at a surprisingly slow rate by the hog kidney enzyme.[3] Using the recombinant enzymes, we have extended this observation with γ-glutamyl peptides containing either (2*S*,4*S*)-4-fluoroglutamate[4] (**2**) or (2*RS*)-4,4-difluoroglutamate (**3**).[5] Preliminary studies were conducted comparing the hydrolysis of AMPte-Glu-γ-Glu (**1**) versus the two analogous fluoroglutamate-containing peptides. The kinetic data indicate that **2** and **3** are hydrolyzed more slowly than **1** by a factor of at least 25 (J. J. Pankuch and J. K. Coward, unpublished results). These data were obtained using a non-continuous end-point HPLC assay.[6] A convenient continuous spectral assay was desired to obtain more accurate kinetic measurements for both the fluoroglutamate-containing substrates and the physiological glutamate-containing substrates. It has been shown that γ-glutamyl peptides lacking the pteridine heterocycle present in folates and methotrexate are excellent GH substrates (e.g., **4**).[6] We now report the synthesis of a similar γ-glutamyl peptide, **5**, containing a fluorescence donor at the N-terminus that is intramolecularly quenched by 3-nitrotyrosine (Tyr(3-NO_2)) at the C-terminus.[7] GH-catalyzed hydrolysis of this peptide results in a time-dependent increase in fluorescence, thereby allowing for the continuous monitoring of the reaction by fluorescence spectroscopy. This is the first internally quenched fluorogenic substrate reported for GH and should enable detailed mechanistic studies as well as more efficient inhibitor screening.

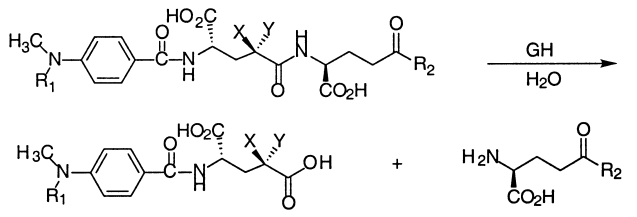
Our approach to the synthesis of the desired peptide is outlined in Scheme 1. This modular approach was chosen to allow for the ultimate incorporation of a wide variety of both natural and unnatural amino acids, e.g., fluoroglutamates, into this type of peptide. Thus, it was envisioned that a differentially protected γ-glutamyl dipeptide could be coupled to the N-terminal fluorophore, *N*-methyl-*p*-aminobenzoic acid (*N*-Me-*p*ABA, **10**) using one of many possible peptide coupling methods. Subsequent selective deprotection of the C-terminal γ-carboxyl group should allow for incorporation of a

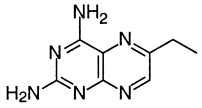
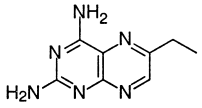
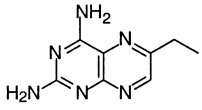
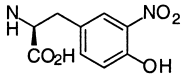
*Corresponding author. Fax: +1-734-647-4865; e-mail: jkcoward@umich.edu

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00103-2

suitably protected 3-nitrotyrosine (**12**) as the chromophore designed to quench the fluorescence of the *N*-Me-*p*AB moiety by intramolecular fluorescence resonance energy transfer (FRET). The differentially protected dipeptide utilizing *tert*-butyl (O*t*Bu) and trimethyl silyl ethyl (OTMSE) esters, Z-Glu(α-O*t*Bu)-γ-Glu(α-O*t*Bu)(γ-OTMSE) (**9**), was synthesized from Z-Glu(α-O*t*Bu)-γ-OH (**8**) and H-Glu(α-O*t*Bu)(γ-OTMSE), the latter compounds derived from the protected oxazolidinone **6**.[8] Removal of the N-terminal Z group (H_2/Pd–C) followed by coupling with *N*-Me-*p*ABA (EDC, HOBt, DMAP) led to the fluorophore-containing peptide, **11**. Selective removal of the TMSE ester was effected with TBAF, and then coupling (EDC, HOBt) of the free γ-COOH to H-Tyr(3-NO_2)-O*t*Bu (**12**) led to the fully protected tripeptide, **13**. Acid hydrolysis with TFA removed all *tert*-butyl esters to provide the desired final product, **5**, after purification by HPLC.[9] All intermediates were completely characterized by NMR (^{1}H, ^{13}C, DEPT) including two-dimensional spectra (COSY, C,H-HETCOR) for selected intermediates.[10] In addition, the final fluorogenic peptide, **5**, was further characterized by LC–MS (see below).

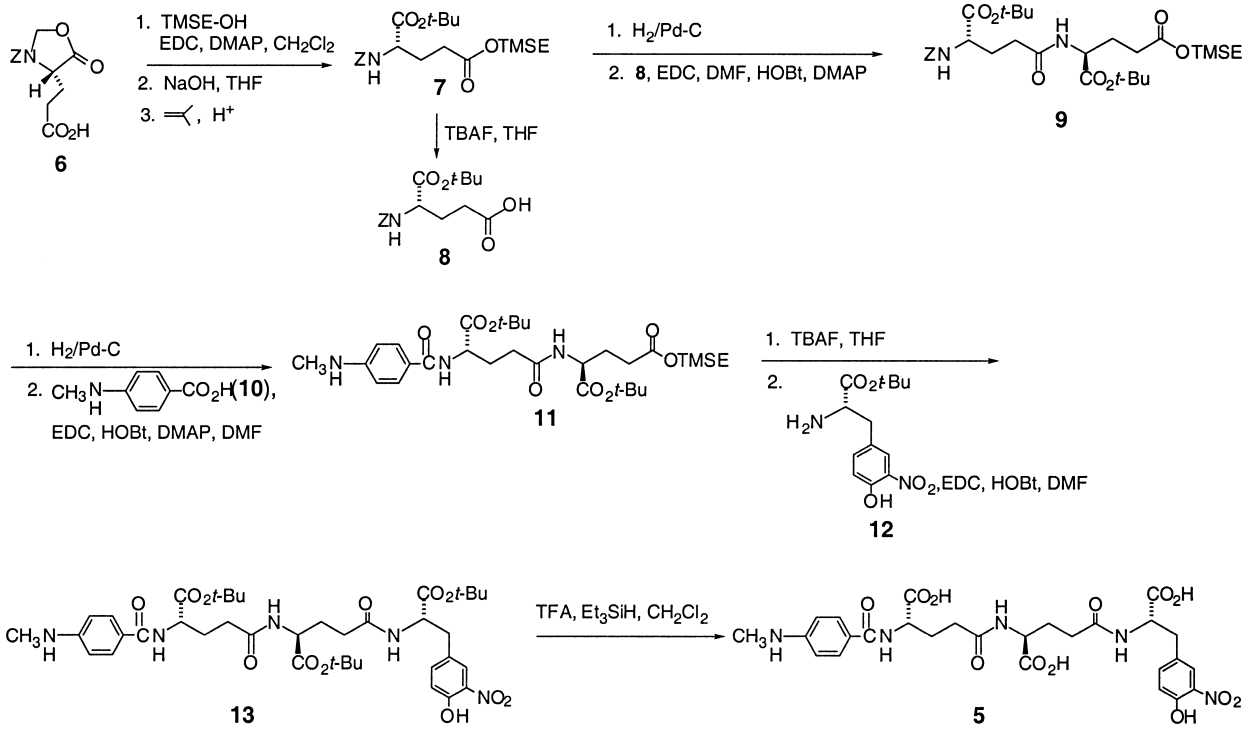
Table 1. Reaction catalyzed by γ-glutamyl hydrolase



Compds	R^1	X	Y	R^2
1		H	H	OH
2		H	F	OH
3[a]		F	F	OH
4	H	H	H	OH
5	H	H	H	

[a]Compound **3** was synthesized as a mixture of racemic diastereomers.

In order to ascertain if peptide **5** is a GH substrate, a three-hour incubation of **5** (100 μM) in the presence of rat GH (rGH)[11] was carried out under standard conditions and the products were analyzed by HPLC.[6,12] Peptide **5** ($t_R = 16.3$ min) was completely hydrolyzed under these conditions and two UV-absorbing products were detected. Spectroscopic analysis of each chromatographic peak (diode array) showed that the early eluting peak contained the *N*-Me-*p*AB chromophore ($\lambda_{max} = 287$ nm, $t_R = 5.2$ min) while the late eluting peak contained the Tyr(3-NO_2) chromophore ($\lambda_{max} = 278$ nm, $t_R = 7.2$ min). Subsequent analysis of the product mixture by LC–MS[13] (Hitachi HPLC components, Finnigan LCQ) clearly identified the products as *N*-Me-*p*AB-Glu-OH ($t_R = 9.05$ min) and H-Glu-γ-Tyr(3-NO_2)-OH ($t_R = 9.37$ min) (Fig. 1A,C). Analysis of the same



Scheme 1.

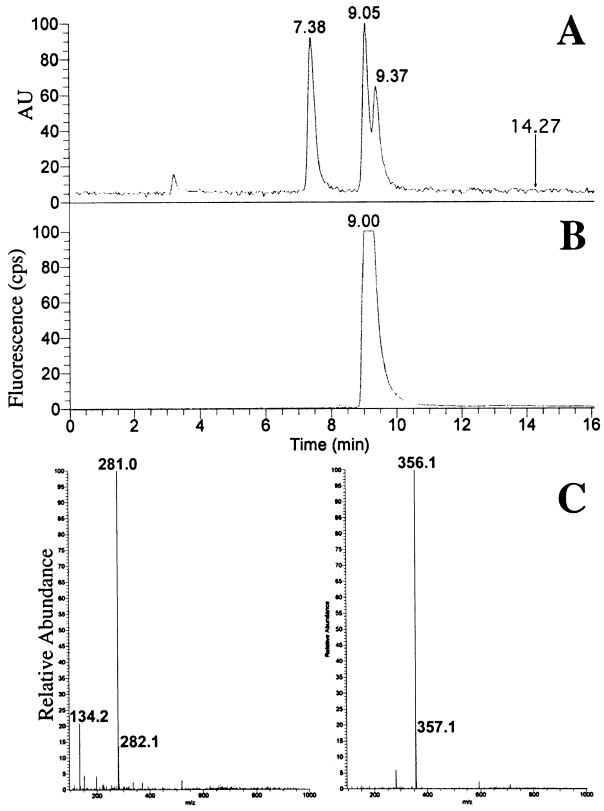


Figure 1. LC–MS analysis of cleavage products produced by extensive incubation with rGH. 50 μM *N*-Me-*p*AB-Glu-γ-Glu-γ-Tyr(3-NO_2) (**5**) was incubated with 3 nM rGH in 100 μL of 50 mM sodium acetate, 50 mM β-mercaptoethanol, pH 6.0 at 37 °C for 3 h. (A) HPLC elution profile by UV (289 nm) where the peak at 7.38 is buffer, and the peaks at 9.05 and 9.37 min are *N*-Me-*p*AB-Glu and Glu-γ-Tyr(3-NO_2), respectively. The arrow at 14.27 min indicates where *N*-Me-*p*AB-Glu-γ-Glu-γ-Tyr(3-NO_2) (**5**) elutes. (B) HPLC elution profile by fluorescence ($\lambda_{ex}=290$, $\lambda_{em}=365$) showing that only the peak at 9.00 min is fluorescent. (C) Mass spectrum of peptide fragment *N*-Me-*p*AB-Glu (left) showing *m*/*z* 281.0 $(M+H)^+$ and Glu-γ-Tyr(3-NO_2) (right) showing $(M+H)^+$ *m*/*z* 356.1. The minor $(M+D)^+$ peaks are due to the presence of deuterium resulting from exposure of **5** to D_2O in prior ^1H NMR experiments.

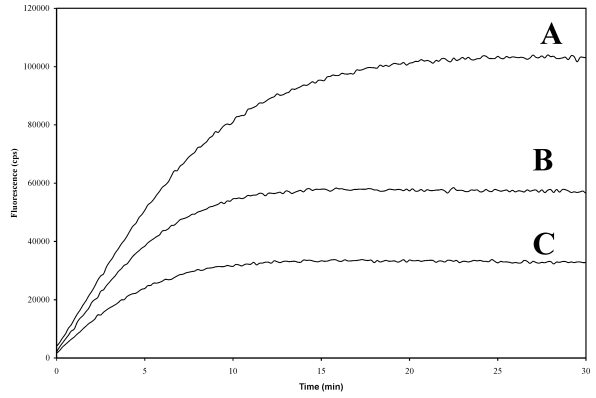


Figure 2. Progress curves for the rat GH-catalyzed hydrolysis of *N*-Me-*p*AB-Glu-γ-Glu-γ-Tyr(3-NO_2) (**5**) (A, 10 μM; B, 5 μM; C, 2.5 μM). GH in storage buffer (25 mM sodium acetate (pH 6.0), 50 mM β-mercaptoethanol, 1 mM β-octylglucoside, 1 mM EDTA, 0.1 M sodium chloride) was preincubated at 37 °C for 30 min. An aliquot (10 μL) of the enzyme solution was added to assay buffer (37 °C) consisting of 50 mM sodium acetate (pH 6.0) and 50 mM β-mercaptoethanol and the indicated final concentration of **5** in a total volume of 100 μL.

samples by fluorescence spectroscopy showed a 7-fold increase in fluorescence upon GH-catalyzed hydrolysis of **5**. In addition, HPLC with fluorescence detection showed that the early peak, identified initially as *N*-Me-*p*AB-Glu based on an identical absorption spectra (diode array) as an authentic synthetic standard, contained the expected fluorophore ($\lambda_{ex}=290$ nm, $\lambda_{em}=365$ nm) (Fig. 1B). These data provide convincing evidence that the GH-catalyzed hydrolysis of **5** proceeds as shown in Table 1.

Further studies of the hydrolysis of **5** by fluorescence spectroscopy demonstrated that the cleavage reaction can be monitored continuously. Incubation of **5** (2.5, 5, 10 μM) with rat GH (3 nM) resulted in a time-dependent increase in fluorescence and an increase in maximum fluorescence with increasing concentration of **5** (Fig. 2). The successful synthesis of **5** and the demonstration that GH-catalyzed hydrolysis of **5** can be monitored continuously in a spectral assay permits the first detailed kinetic investigation of this key enzyme in folate biochemistry. In addition, the spectral assay will greatly facilitate the evaluation of mechanism-based inhibitors of GH, the synthesis of which is currently underway in our laboratory.

Acknowledgements

This research was supported by a grant from the National Cancer Institute (CA28097). JJP is a trainee of the Michigan Chemistry–Biology Interface Training Program and was supported in part by a grant from the National Institutes of General Medical Sciences (GM08597). We thank Drs. John Galivan and Thomas Ryan for generously providing the recombinant GH used in this research and for many stimulating discussions. We thank Matthew Alexander for helpful discussions concerning the synthesis of **5**, David Rosen for his work on the fluorescence assay, and Prof. Kevin Rice and Dr. Donald McKenzie for LC–MS analysis of the products obtained from GH-catalyzed hydrolysis of **5**.

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9. HPLC: Vydac C18 column, gradient conditions were 3% solvent A (acetonitrile) for 1 min (solvent B 0.1 M NH_4OAc, pH 5.5) followed by an increase to 25% solvent A over 25 min at 1 mL/min.

10. Selected ^1H and ^{13}C NMR spectroscopic data. **5**: ^1H NMR (D_2O, 500 MHz) δ 7.84 (s, 1H), 7.62–7.58 (d, 2H), 7.44 (s, 1H), 6.99–6.96 (d, 1H), 6.68–6.67 (d, 2H), 4.39–4.34 (m, 2H), 3.95 (m, 1H), 3.17–3.12 (m, 2H), 2.87–2.84 (d, 1H), 2.75 (s, 3H), 2.38–1.70 (m, integration obscured by NH_4OAc peak, δ 1.97). **7**: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.34–7.31 (m, 5H), 5.43–5.40 (d, 1H, NH), 5.09 (s, 2H), 4.29–4.24 (t, 1H), 4.18–4.10 (t, 2H), 2.35–1.92 (m, 4H), 1.45 (s, 9H), 0.99–0.94 (t, 2H), 0.00 (s, 8H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.0, 171.1, 156.0, 136.4, 128.6 (CH), 128.2 (CH), 82.5, 67.0 (CH_2), 63.0 (CH_2), 54.0 (CH), 30.8 (CH_2), 28.1 (CH_3), 17.4 (CH_2), −1.4 (CH_3). Purification of **7** by silica gel chromatography led to a product which was slightly contaminated by Z-Glu($OtBu$)$_2$, resulting from incomplete TMSE protection of the γ-CO_2H of **6** and/or partial hydrolysis of the intermediate γ-TMSE ester prior to reaction with isobutylene. **8**: ^1H NMR ($CDCl_3$, 300 MHz) δ 10.71 (s, 1H), 7.37–7.28 (m, 5H), 5.43–5.41 (d, 1H), 5.11 (s, 2H), 4.34–4.28 (m, 1H), 2.48–2.21 (m, 2H), 2.21–2.17 (m, 1H), 1.98–1.91 (m, 1H), 1.48 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 178.2, 171.1, 156.2, 136.3, 128.7 (CH), 128.4 (CH), 128.3 (CH), 82.9, 67.3 (CH_2), 53.8 (CH), 31.8 (CH_2), 30.1 (CH_2), 28.1 (CH_3). **9**: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.36–7.31 (m, 5H), 6.43 (d, 1H), 5.58 (d, 1H), 5.12 (s, 2H), 4.49–4.48 (d, 1H), 4.24 (d, 1H), 4.18–4.15 (m, 2H), 2.35–2.28 (m, 6H), 1.95 (m, 2H) 1.46–1.44 (d, 18H), 0.99–0.96 (m, 2H), 0.00 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.1, 172.0, 171.1, 156.3, 136.3, 128.6 (CH), 128.2 (CH), 82.5, 67.1 (CH_2), 63.0 (CH_2), 54.0 (CH), 53.5, 52.3 (CH), 32.3 (CH_2), 30.5 (CH_2), 28.9 (CH_2), 28.1 (CH_3), 27.7 (CH_2), 17.3 (CH_2), −1.4 (CH_3). COSY and C,H-HETCOR spectra were also collected. **11**: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.67–7.64 (d, 2H), 7.13–7.10 (d, 1H), 6.98–6.96 (d, 1H), 6.53–6.50 (d, 2H), 4.58 (m, 1H), 4.47–4.45 (m, 1H), 4.15–4.10 (m, 2H), 2.80 (s, 3H), 2.40–1.96 (m, 8H + EtOAc), 1.44–1.41 (d, 18H), 0.97–0.91 (t, 2H), 0.00 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.9, 172.3, 171.6, 171.1, 170.9, 167.3, 152.2, 128.8 (CH), 121.2 (CH), 111.2 (CH), 82.1, 62.7 (CH_2), 52.6 (CH), 52.2 (CH), 32.5 (CH_2), 30.4 (CH_2), 30.1 (CH_3), 28.6 (CH_2), 27.9 (CH_3), 27.4 (CH_2), 17.2 (CH_2), −1.6 (CH_3). COSY and C,H-HETCOR spectra were also collected. **12**: ^1H NMR (CD_3OD, 300 MHz) δ 7.82 (s, 1H), 7.39–7.35 (m, 1H), 6.99–6.96 (d, 1H), 3.55–3.50 (m, 1H), 2.85–2.83 (m, 2H), 1.27 (s, 9H); ^{13}C NMR (CD_3OD, 75 MHz) δ 174.4, 154.4, 139.1 (CH), 135.7, 129.8, 126.7 (CH), 121.4 (CH), 82.9, 56.8 (CH), 40.1 (CH_2), 28.2 (CH_3). **13**: ^1H NMR ($CDCl_3$, 300 MHz) δ 10.40 (s, 1H), 7.81–7.80 (d, 1H), 7.67–7.64 (d, 2H), 7.38–7.35 (m, 2H), 7.00–6.97 (d, 1H), 6.89–6.86 (d, 1H), 6.69–6.67 (d, 1H), 6.54–6.51 (d, 2H), 4.70–4.65 (m, 2H), 4.44 (m, 1H), 2.99–2.92 (m, 2H), 2.87 (s, 3H), 2.41–1.84 (m, 11H), 1.50–1.38 (m, 31H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.3, 171.8, 171.8, 170.9, 170.2, 167.4, 153.9, 152.2, 138.9 (CH), 133.0, 129.2, 128.9 (CH), 125.2 (CH), 121.1, 119.7 (CH), 111.3 (CH), 82.6, 82.3, 53.5 (CH), 52.2 (CH), 52.0 (CH), 36.6 (CH_2), 32.4 (CH_2), 32.0 (CH_2), 30.1 (CH_3), 29.6 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 28.0 (CH_3), 27.9 (CH_3), 0.4 (CH_3). COSY and C,H-HETCOR spectra were also obtained.

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12. HPLC: Vydac C18 column, gradient conditions were 3% solvent A (acetonitrile) for 3 min (solvent B 0.1 M NaOAc, pH 5.5) followed by an increase to 4% solvent A over 5 min, followed by an increase to 95% solvent A over 5 min at 1 mL/min.

13. HPLC: Vydac C18 column, gradient conditions were 3–50% solvent B (solvent A: H_2O, 0.1% AcOH, 0.02% TFA; solvent B: CH_3CN, 0.1% AcOH, 0.02% TFA).