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Transition State Isosteres of the γ-Glutamyl Peptide Bond Hydrolysis: Synthesis and Characterization of the $\psi(CH_2NH)$ Pseudopeptide Analogue of Glutathione

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Abstract: The fully deprotected glutathione analogue containing the aminomethylene unit as transition state isostere of the γ-Glu-Cys peptide bond was synthesized for the first time and characterized in both the reduced and oxidized forms. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: γ-glutamyl peptide bond; glutathione; reduced peptides; transition state analogue

INTRODUCTION

Due to its biochemical relevance, attention is being focused on the chemical and medical aspects of the glutathione molecule (γ-L-glutamyl-L-cysteinyl-glycine; γ-Glu-Cys-Gly) in both its reduced thiol (GSH) and oxidized disulfide (GSSG) forms [1–5]. Accordingly, a variety of glutathione derivatives and analogues has been described. These analogues include amino acid substitutions and peripheral alterations as well as the introduction of non-peptide bonds into the backbone, resulting in the formation of pseudopeptide species [6–8].

In this connection a strategy to replace the enzymatically scissile peptide bonds with stable isosteres is at present being pursued in order to obtain models characterized by metabolic stability as well as improved ligand–acceptor recognition and binding [9–13].

In the cited field, a new group of glutathione analogues were reported containing urea [14] or urethane [15] groups replacing the native γ-glutamyl isopeptide bond, which is the cleavage site of γ-glutamyl transpeptidase (γ-GT) [16–20] as well as a structural determinant in GSH recognition and binding to biomacromolecules [21,22]. Both these amide bond surrogates present stability towards enzymatic hydrolysis and maintain a planar structure at the key site; therefore, the sp^3 aminomethylene CH_2NH group was of interest to mimic the tetrahedral intermediate of γ-Glu-Cys peptide bond hydrolysis [23]. In fact, among the transition state analogues (TSAs) of the cleavable peptide bonds [24–29], the $\psi[CH_2NH]$ isosteric replacement introduces unique properties within the peptide sequence other than the expected enzyme resistance and increased flexibility, i.e. a new base, a new ionizable site and increased H-bond donor ability.

A valuable contribution has recently been given by Burg *et al.* while studying γ-GT stable glutathione S-transferase inhibitors [30]. They reported synthesis and properties of ethacrynic acid-GSH S-conjugates characterized by the presence of a reduced γ-Glu-Cys amide bond. However, only fully protected

Abbreviations: Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature as given in *Eur. J. Biochem* 1984; **138**: 9–37. Abbreviations listed in the guide recently published in *J. Peptide Sci.* 1999; **5**: 465–471 are used without explanations. Additional abbreviations: DIBAL, diisobutylaluminium hydride; HETCOR, heteronuclear chemical shift correlation.

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derivatives of the polyfunctional ψ[CH_2NH] GSH and GSSG peptides have been described by these authors. In view of the chemical and biochemical relevance of the free species, with particular reference to the thiol-disulfide redox system, an alternative and efficient synthesis of the fully protected forms and the first chemical characterization of the free forms of ψ[CH_2NH] GSH and ψ[CH_2NH] GSSG, are reported.

MATERIALS AND METHODS

TLC was performed on Merck 60 F_{254} silica gel plates developed with the following solvent systems: (a) $CHCl_3$/MeOH (95 : 5); (b) $CHCl_3$/MeOH (97 : 3); (c) $CHCl_3$/*i*-PrOH (95 : 5); (d) *n*-BuOH/AcOH/H_2O (2 : 4 : 4). Column chromatography was carried out using Merck 60 silica gel (230–400 mesh). Optical rotations were taken at 25 °C with a Perkin-Elmer 241 polarimeter. IR absorption spectra were recorded employing a Perkin-Elmer 1600 FTIR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300 MHz instrument (δ expressed in ppm). The assignments of the ^{1}H- and ^{13}C-NMR resonances of analogues **4**–**8** were confirmed using standard 2D NMR spectroscopic techniques (HETCOR). The mass spectra of **7** and **8** were obtained under electrospray (ES) conditions by a LCQ (Finnigan, San Josè, CA, USA) instrument. The 60 µM solution of the sample in a 50 : 50 CH_3OH/H_2O solvent mixture containing 1% AcOH was directly injected at the flow rate of 5 µl/min.

N,N-di-Boc-γ-Glu-OtBu-ψ(CH_2NH)-Cys(Acm)-Gly-OtBu (4)

To a stirred solution of (*S*)-(-)-1-*tert*-butyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-oxopentanoate (**3**) [31] (5.0 g, 12.9 mmol) and H-Cys(Acm)-Gly-OtBu [15] (3.95 g, 12.9 mmol) in MeOH containing 1% of AcOH (135 ml) $NaBH_3CN$ (2.43 g, 38.7 mmol) was added portionwise at room temperature over a period of 30 min. After an additional 1.5 h, the solvent was removed under reduced pressure, the residue taken up in EtOAc and the organic layer washed with saturated aqueous $NaHCO_3$ and H_2O. The residue obtained after drying over $MgSO_4$ and evaporation was chromatographed on silica gel using $CHCl_3$/MeOH (95 : 5) as eluant to give tripeptide di-*tert*-butyl ester **4** as an oil. Yield: 6.20 g (71%); R_f(a) = 0.43; $[\alpha]_D = -4.8°$ ($c = 1$, $CHCl_3$); IR ($CHCl_3$): $\upsilon = 3340$ br, 1740, 1650, 1540 cm^{-1}; ^{1}H-NMR: ($CDCl_3$): $\delta = 1.38$ and 1.43 (36H, 2 × s, 12 × CH_3), 1.55 (2H, m, Glu γ-CH_2), 1.88 (1H, m, Glu β-CH_A), 1.90 (1H, obs, CH_2NH), 1.99 (3H, s, Acm CH_3), 2.12 (1H, m, Glu β-CH_B), 2.55 (1H, m, Glu δ-CH_A), 2.67 (1H, m, Glu δ-CH_B), 2.92–3.09 (2H, m, Cys β-CH_2), 3.30 (1H, m, Cys α-CH), 3.91 (2H, m, Gly CH_2), 4.29 (2H, m, Acm CH_2), 4.72 (1H, m, Glu α-CH), 6.92 (1H, t, $J = 5.5$ Hz, Acm NH), 7.93 (1H, t, $J = 5.7$ Hz, Gly NH); ^{13}C-NMR: ($CDCl_3$): $\delta = 23.39$ (Acm CH_3), 27.08 (Glu C^β), 27.20 (Glu C^γ), 28.13, 28.24 (Boc, tBu), 32.89 (Cys C^β), 40.94 (Acm CH_2), 41.96 (Gly CH_2), 48.20 (Glu C^δ), 58.78 (Cys C^α), 61.32 (Glu C^α), 81.49, 82.39, 83.00, 83.10 (4 × Cq, Boc, tBu), 152.78 (2 × Boc CO), 168.93, 170.03, 170.46 and 174.00 (4 × CO).

N,N-di-Boc-γ-Glu-OtBu-ψ(CH_2N-Boc)-Cys(Acm)-Gly-OtBu (5)

DIPEA (1.95 ml, 8.9 mmol) and Boc_2O (3.54 g, 16.24 mmol) were added to a solution of the reduced peptide isostere **4** (5.50 g, 8.12 mmol) in dry acetonitrile (245 ml). After 48 h at 60 °C under stirring, the reaction mixture was evaporated under vacuum and the residue obtained was purified on silica gel using $CHCl_3$/MeOH (97 : 3) as eluant to yield the pure tripeptide di-*tert*-butyl ester **5** as a foam. Yield: 5.67 g (90%); R_f(b) = 0.50; $[\alpha]_D = -37.5°$ ($c = 1$, $CHCl_3$); IR ($CHCl_3$): $\upsilon = 3400$ br, 1740, 1690, 1535 cm^{-1}; ^{1}H-NMR: (DMSO-d_6): $\delta = 1.41$ and 1.44 (45H, 2 × s, 15 × CH_3), 1.50–1.88 (4H, m, Glu β- and γ-CH_2), 1.88 (3H, s, Acm CH_3), 2.71 (1H, m, Cys β-CH_A), 2.98–3.14 (3H, m, Cys β-CH_B and Glu δ-CH_2), 3.67 (2H, m, Gly CH_2), 4.02 (1H, m, Acm CH_A), 4.32 (1H, m, Acm CH_B), 4.58 (1H, m, Glu α-CH), 4.68 (1H, m, Cys α-CH), 8.18 (1H, t, $J = 5.0$ Hz, Gly NH), 8.47 (1H, t, $J = 5.0$ Hz, Acm NH); ^{13}C-NMR: (DMSO-d_6): $\delta = 23.23$ (Acm CH_3), 26.59 (Glu C^γ), 28.21, 28.63 (Boc, tBu), 29.20 (Glu C^β), 30.89 (Cys C^β), 40.20 (Acm CH_2), 42.23 (Gly CH_2), 44.61 (Glu C^δ), 57.82 (Glu C^α), 58.86 (Cys C^α), 79.81, 81.17, 81.31, 82.81, 83.08 (5 × Cq, Boc, tBu), 152.32, 152.68 (3 × Boc CO), 169.32, 169.69, 170.05 and 170.53 (4 × CO).

(*N,N-di*-Boc-γ-Glu-OtBu-ψ(CH_2N-Boc)-Cys-Gly-OtBu)$_2$ (6)

To a stirred solution of the above reported tripeptide di-*tert*-butyl ester **5** (5.0 g, 6.4 mmol) in MeOH (66 ml), I_2 (3.25 g, 12.8 mmol) in MeOH (33 ml) was added portionwise at room temperature during 30 min. After 30 min under stirring the reaction mixture was cooled at 0 °C and decolorized with 1 N

$Na_2S_2O_3$. The residue obtained after removal of the solvent was partitioned between H_2O and EtOAc and the organic layer was washed with 1 N $Na_2S_2O_3$ and H_2O. Drying over Na_2SO_4 and evaporation followed by purification by silica gel column chromatography ($CHCl_3$/*i*-PrOH 95 : 5 as eluant) of the resulting crude product afforded the corresponding pure disulfide ester **6** as a foam. Yield: 3.06 g (34%); R_f(c) = 0.66; $[\alpha]_D = -55.7^\circ$ ($c = 1$, $CHCl_3$); IR ($CHCl_3$): $\upsilon = 3390$ br, 1740, 1695, 1520 cm^{-1}; ^{1}H-NMR: (DMSO-d_6): $\delta = 1.35–1.47$ (90H, br s, $30 \times CH_3$), 1.50–1.90 (8H, m, Glu γ-CH_2 and β-CH_2), 2.91 (2H, m, Cys β-CH_A), 3.05 (2H, m, Glu δ-CH_A), 3.28 (2H, m, Cys β-CH_B), 3.30 (2H, m, Glu δ-CH_B), 3.68 (4H, m, Gly CH_2), 4.59–4.70 (4H, m, Cys α-CH and Glu α-CH), 8.26 (2H, br t, Gly NH); ^{13}C-NMR: (DMSO-d_6): $\delta = 26.98$ (Glu C^γ), 28.20, 28.84 (Boc, tBu), 28.98 (Glu C^β), 30.65 (Cys C^β), 42.29 (Gly CH_2), 44.70 (Glu C^δ), 58.28 (Glu C^α), 58.80 (Cys C^α), 81.13, 81.25, 81.38, 82.76, 83.06 ($10 \times$ Cq, Boc, tBu), 152.56, 152.69 ($6 \times$ Boc CO), 169.24, 169.37 and 170.00 ($6 \times$ CO).

(γ-Glu-ψ(CH_2NH)-Cys-Gly)$_2$ (7)

The protected disulfide **6** (2.50 g, 1.77 mmol) was dissolved in 18 ml TFA/370 µl H_2O. After 6 h under stirring at room temperature, the reaction mixture was evaporated *in vacuo* to dryness. The residue was repeatedly washed with diethyl ether, taken up in H_2O and the pH of the resulting aqueous solution adjusted to 6.0 using 1 N NaOH. The solution was concentrated and subjected to column chromatography on Sephadex LH-20 using H_2O/MeOH 2 : 1 as eluant to afford compound **7** as a vitrous foam. Yield: 0.88 g (85%); R_f(d) = 0.54; $[\alpha]_D = -29.3^\circ$ ($c = 1$, H_2O); IR (KBr): $\upsilon = 3240$, 1680–1530 cm^{-1}; MS (m/z): 585 $(M + H)^+$.

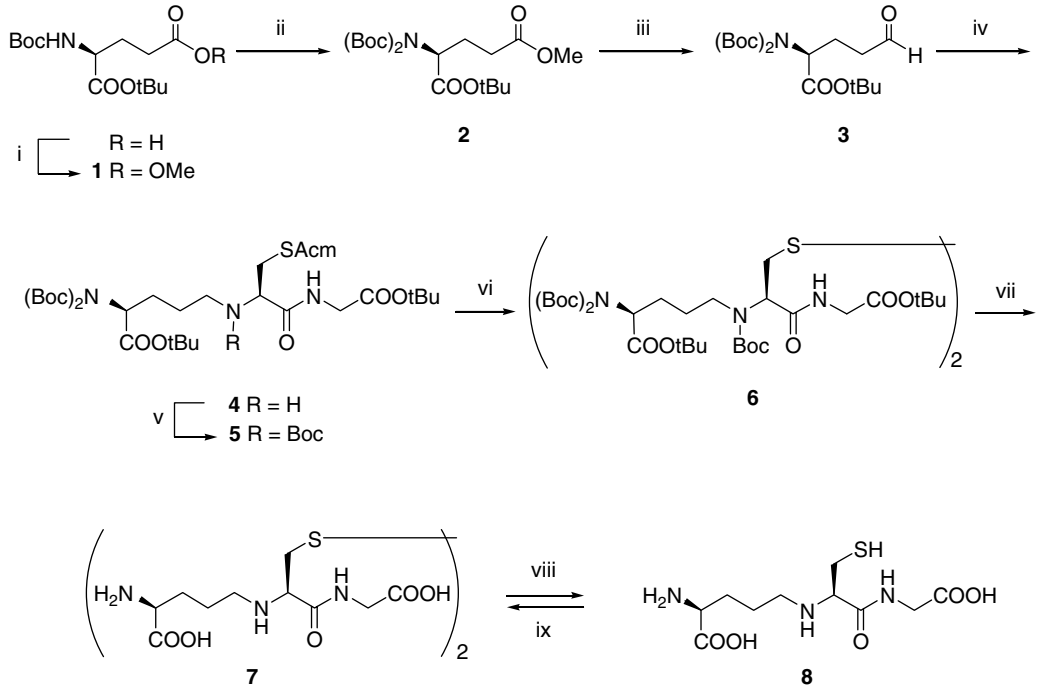
Compound **7** has been also obtained starting from **8**: to a stirred solution of **8** (0.20 g, 0.68 mmol) in H_2O (10 ml) brought to pH 8.5 with 25% aqueous NH_3, O_2 was bubbled. After 4 h at room temperature, the solution was concentrated and subjected to column chromatography on Sephadex LH-20 (H_2O/MeOH 2 : 1 as eluant) to give title compound **7** in 80% yield.

γ-Glu-ψ(CH_2NH)-Cys-Gly (8)

A solution of the foregoing disulfide **7** (0.81 g, 1.38 mmol) in a mixture of *n*-PrOH/H_2O (2 : 1) (15 ml) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. Tri-*n*-butylphosphine (0.34 g, 1.68 mmol) was added and the stoppered flask stirred at room temperature. After 1.5 h the reaction mixture was repeatedly washed with $CHCl_3$ and the pH of the aqueous solution adjusted to 6.0 using 1 N HCl. The solution was concentrated and subjected to column chromatography on Sephadex LH-20 using H_2O/MeOH (2 : 1) as eluant to afford the corresponding reduced compound **8**. Yield: 0.39 g (97%); R_f(d) = 0.44; $[\alpha]_D = -35.5^\circ$ ($c = 1$,H_2O); IR (KBr): $\upsilon = 3390$ br, 1670–1550 cm^{-1}; MS (m/z): 294 $(M + H)^+$.

RESULTS AND DISCUSSION

The target pseudopeptides **7** and **8** were synthesized using solution procedures as outlined in Scheme 1. The critical step is represented by the incorporation of the reduced peptide bond at the γ-Glu-Cys site. To this end the most obvious way would envision reductive alkylation of the amino component with the appropriate amino acid aldehyde [32]. This approach was investigated by Burg *et al.* [30], but it was discarded owing to difficulties encountered during the oxidation of the precursor γ-glutaminol derivative to the corresponding key aldehyde requested for the condensation reaction. An alternative route is reported involving reduction of the γ-carboxylate group of the suitably protected glutamic acid derivative **2** (Scheme 1). By following the procedure of Adamczyk *et al.* [31], the commercially available Boc-Glu(OH)-OtBu was converted to the corresponding methyl ester **1** by reaction with methyl chloroformate in the presence of TEA and catalytic amounts of DMAP. Compound **1** was derivatized prior to the reduction step by introducing an additional *N*-Boc protecting group, in order to avoid cyclization of the urethane nitrogen onto the newly generated aldehyde function [33]. *N*,*N*-di-*tert*-butyloxycarbonyl-α-*tert*-butyl L-glutamate γ-semialdehyde (**3**) was thus prepared without significant side reactions upon treatment of **2** with DIBAL in diethyl ether at −78 °C, and reductively coupled with the building block H-Cys(Acm)-Gly-OtBu [15] by using $NaBH_3CN$ (3 equiv.) in MeOH containing 1% AcOH to afford the protected pseudotripeptide **4** in a good yield. Selective removal of the Acm group from the thiol function with iodine in MeOH was performed on the *N*-Boc derivatized pseudotripeptide **5**, affording smooth conversion to the symmetrical disulfide **6**. Acidolytic cleavage in a single step of all five protecting groups of compound **6**, followed



Scheme 1 Reagents/Conditions: (i) $ClCO_2Me/DMAP/Et_3N/CH_2Cl_2/0\,^{\circ}C$ 1 h; (ii) $Boc_2O/DMAP/CH_3CN$/r.t. 24 h; (iii) $DIBAL/Et_2O/-78\,^{\circ}C$ 15 min; (iv) H-Cys(Acm)-Gly-OtBu, $NaBH_3CN$/MeOH-1% AcOH/r.t. 2 h; (v) Boc_2O/DIPEA/MeCN/60 °C 48 h; (vi) I_2/MeOH/r.t. 1 h; (vii) TFA/r.t. 6 h, then 1N NaOH; (viii) $(n\text{-}Bu)_3P/n\text{-}PrOH : H_2O$ 2 : 1/r.t. 1.5 h; (ix) O_2, NH_3, r.t. 4 h.

by treatment of the resulting *tetra*-trifluoroacetate with aqueous 1 N NaOH (pH 8.5), gave the fully deprotected GSSG pseudopeptide analogue **7**. The desired GSH thiol form **8** was finally obtained in high yields by reductive cleavage of the disulfide link with tri-*n*-butylphosphine, following an our previously published procedure [14]. As expected the thiol **8** can be converted into the starting disulfide **7** by oxidation in the presence of O_2. The two peptides were purified to homogeneity and characterized by optical rotation, IR absorption, NMR and mass spectrometry. The structures assigned to the glutathione analogues **7** and **8** are in accordance with their spectroscopic properties. Mass spectra of **7** and **8** show the most abundant peaks corresponding to $M + H^+$ species (585 and 294 m/z, respectively). ^{1}H- and ^{13}C-NMR analyses of the two products reveal only one set of signals, thus confirming the absence of diastereoisomers. A characteristic feature of the ^{13}C-NMR spectrum of **8** (see Table 1) is represented by the resonance of the Cys β-carbon atom which is shifted at higher field (25.88 ppm) compared with the corresponding atom in the disulfide precursor **7** (39.74 ppm). This spectral behaviour represents, as recently noted [34], a reliable diagnostic feature

Table 1 ^{1}H- and ^{13}C-NMR Data[a] for Compounds **7** and **8**

Residue	(γ-Glu-ψ[CH_2NH]-Cys-Gly)$_2$ (**7**)		γ-Glu-ψ[CH_2NH]-Cys-Gly (**8**)	
	δ_H	δ_C	δ_H	δ_C
Glu				
C^α	3.55 *m*	60.65	3.57 *m*	63.09
C^β	1.74 *m*	28.84	1.74 *m*	28.46
C^γ	1.42 *m*	24.78	1.49 *m*	24.06
C^δ	2.48 *m*	46.62	2.57 *m*	46.25
CO	—	174.16[b]	—	172.76[c]
Cys				
C^α	3.46 *m*	54.68	3.39 *m*	54.52
C^β	2.76–2.94 *m*	39.74	2.68 *m*	25.88
CO	—	175.11[b]	—	174.81[c]
Gly				
C^α	3.62 *q* (16.0)	43.60	3.62 *q* (16.5)	43.43
CO	—	176.58[b]	—	176.45[c]

[a] In D_2O at 25 °C; J/Hz in parentheses; *m* and *q* refer to spin multiplicity (*m*, multiplet; *q*, quartet).
[b,c] Assignments may be interchanged.

in order to distinguish between cysteine and cystine containing forms.

CONCLUSIONS

An efficient and straightforward strategy for the synthesis of GSH and GSSG pseudopeptides characterized by the presence of a reduced γ-Glu-Cys amide bond has been described by adopting the reductive amination protocol.

Introduction of a peptide bond surrogate lacking the amide carbonyl, while featuring a secondary amine group, is expected to have profound conformational effects on the GSH structure, mainly connected with increased flexibility and an altered hydrogen-bond pattern. The presence of this uncleavable junction on both fully protected and free forms represents, on the other hand, a useful starting point for the development of further *in vivo* long-lasting GSH analogues. Furthermore, the character of transition state isostere of the γ-Glu-Cys bond hydrolysis, typical of the $\psi[CH_2NH]$ bond, renders these pseudopeptides promising substrates for a variety of glutathione-dependent enzymes. Studies in this field are being performed in our laboratory.

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