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Novel glutathione analogues containing the dithiol and disulfide form of the Cys-Cys dyad

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Summary. The glutathione analogue γ-(H-Glu-OH)-$\overline{\text{Cys-Cys}}$-OH (**5**), containing the 8-membered disulfide ring -$\overline{\text{Cys-Cys}}$ replacing the native -Cys-Gly fragment, has been synthesized and characterized together with its reduced dithiol form γ-(H-Glu-OH)-Cys-Cys-OH (**6**).

Keywords: Amino acids – Conformational constraint – Cyclic disulfides – -$\overline{\text{Cys-Cys}}$-peptides – Dipeptide mimetics – Dithiols – Glutathione analogues

Abbreviations: Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature as given in *Eur. J. Biochem.* (1984) 138, 9–37. Additional abbreviations: DBU, 1,8-diazabicyclo [5.4.0] undec-7-ene; DCCI, *N,N'*-dicyclohexylcarbodiimide; NMM, *N*-methylmorpholine; THF, tetrahydrofuran; $(n\text{-Bu})_3\text{P}$, tri-*n*-butylphosphine

Introduction

In the research area of drug design much effort is focused on the development of mimetics of bioactive natural peptides with improved therapeutical potential. In several cases the chemical modification strategy relies upon the replacement of a dipeptide fragment with a conformationally constrained framework suitable to stabilize specific backbone conformations and limit the rotational space of the side chains (Shao et al., 1996; Shreder et al., 1998). Conformational analysis of a representative collection of dipeptide mimetics (Gillespie et al., 1997) together with reviews on this subject (Goodman et al., 1997; Hanessian et al., 1997) have been recently published and confirm the growing interest in this area.

In the context of our studies on the synthesis of glutathione (GSH) analogues (Luisi et al., 1993; Calcagni et al., 1995; Calcagni et al., 1996) we are investigating structures containing cyclic frameworks centered at the *C*-terminal moiety. Although a number of chemical modifications have been performed on this molecule (Chen et al., 1986; Douglas, 1989; Xie et al., 1991; Embrey et al., 1994; Gröger et al., 1996), literature examination revealed that no data are available on GSH analogues containing a Cys residue replacing the *C*-terminal Gly and giving rise to a -Cys-Cys sequence.

Although the Cys-Cys diad occurs commonly in proteins, it rarely gives rise to the 8-membered cyclic disulfide -$\overline{\text{Cys-Cys}}$-, in which the two adjacentresidues are connected by both peptide and disulfide linkages. This constrained building block, associated with the redox system, represents however a well documented critical sequence in some proteins (Ovchinnikov et al., 1985) where it can act as a molecular switch able to control receptor (Kao et al., 1986) or enzyme (Miller et al., 1989; White et al., 1993) activation; its presence in natural peptides is also known (Mitra et al., 1984).

Thus, the Cys *versus* Gly replacement into the glutathione system appears very attractive for more than one reason: possible participation to redox equilibria involving intra- or inter-molecular disulfide forms (Izumiya et al., 1954; Capasso et al., 1984; Siedler et al., 1993); topographical control of the sulfurated central side chain when covalently bonded to the adjacent cysteine (Brady et al., 1993); biochemical and conformational consequences derived by the preferred *cis*-conformation adopted by the CONH bond when inserted into an eight-membered disulfide ring (Capasso et al., 1979; Sukumaran et al., 1991; Brady et al., 1993; Liff et al., 1996). It is worth mentioning here that the conformational properties of the -$\overline{\text{Cys-Cys}}$- peptides have been the object of detailed studies since the potential energy calculations of Chandrasekaran et al. (1969) and the X-ray crystallographic analysis of Mez (1974), Hata et al. (1977) and Capasso et al. (1977). More recently the conformation of this highly constrained cyclic dipeptide has been investigated by several authors both in the form of isolated fragment (Horne et al., 1993; Cumberbatch et al., 1993) and as making part of a linear (Prorok et al., 1990; Avizonis et al., 1996; Liff et al., 1996) or cyclic oligopeptide system (Brady et al., 1993).

On the basis of the above considerations, it seemed interesting to examine a GSH-related system characterized by the presence of the *C*-terminal Cys-Cys dyad in its reduced and conformationally constrained oxidized form and here we report synthesis and characterization of γ-(H-Glu-OH)-$\overline{\text{Cys-Cys}}$-OH (**5**) and γ-(H-Glu-OH)-Cys-Cys-OH (**6**).

Material and methods

TLC was performed on Merck 60 F$_{254}$ silica gel plates developed with the following solvent systems: (a) CHCl$_3$:hexane 8:2 by vol.; (b) CHCl$_3$:hexane 9:1 by vol.; (c) CHCl$_3$:diethyl ether 96:4 by vol.; (d) CHCl$_3$:MeOH 97:3 by vol.; (e) *n*-BuOH:AcOH:H$_2$O 4:5:1 by vol.; (f)*n*-BuOH:AcOH:H$_2$O 6:4:4 by vol.; (g) phenol:H$_2$O 4:1 w/v. HPLC was performed with a Waters system equipped with a 680 gradient controller and a 996 photodiode array detector linked to a Millenium 2010 data station; column: Symmetry C-18 reversed-phase (250 × 4.6 mm) with 5 μm packing material; the solvent was 50 mM aqueous formic acid at flow rate of 1.2 ml/min.; detection at 226 nm. Column chromatography was carried out using Merck 60 silica gel (230–400 mesh). Optical rotations were taken at 20°C with a Schmidt-Haensch Polartronic D polarimeter. IR spectra were recorded employing a Perkin-Elmer 983 spectrophotometer. All compounds gave correct elemental analyses for C, H, N, and S (performed on a Carlo Erba model 1106 analyzer). ^{1}H-(300 MHz) and ^{13}C-(75.43 MHz) NMR spectra were determined on a Varian XL-300 instrument. ^{13}C-NMR chemical shifts were measured relative to internal dioxane (67.40 ppm) for compounds **5** and **6**, and relative to internal tetramethylsilane for the other compounds. The mass spectra of **5** have been obtained in electrospray (ES) conditions by a LCQ (Finnigan, S. José, CA, USA) instrument. The 3 nM solution of the sample in $CH_3CN:H_2O$ 50:50 containing 0.1% formic acid has been directly injected at a flow rate of 8 μl/min.

Boc-Glu(OH)-O*t*Bu, H-Cys(Trt)-O*t*Bu and Fmoc-Cys(Trt)-O*t*Bu were obtained from Novabiochem.

*Fmoc-Cys(Trt)-Cys(Trt)-OtBu (**1**)*

To a stirred solution of Fmoc-Cys(Trt)-OH (5.15 g, 8.8 mmol) and H-Cys(Trt)-O*t*Bu (3.7 g, 8.8 mmol) in dry CH_2Cl_2 (50 mL) DCCI (1.8 g, 8.8 mmol) in CH_2Cl_2 (10 mL) was added portionwise at 0°C. After 2 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the resulting solution washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$ and H_2O. The residue obtained after drying and evaporation was chromatographed on silica gel using $CHCl_3$:hexane (8:2) as eluant to give *tert*-butyl ester **1** as a foam (7.8 g, 90%). – $[\alpha]$D + 2.0° (c 2, $CHCl_3$). – Rf (a) 0.45 – IR ($CHCl_3$): 3410, 1730, 1675, 1490 cm^{-1}. – ^{1}H-NMR ($CDCl_3$) δ = 1.42 (9H, s, 3 × CH_3), 2.53 (2H, m, Cys β-CH_2, 2.69 (2H, m, Cys β-CH_2), 3.88 (1H, Cys α-CH), 4.15–4.45 (3H, m, Fmoc CH-CH_2), 4.33 (1H, Cys α-CH), 5.02 (1H, d, J = 7.0 Hz, NH-COO), 6.48 (1H, d, J = 7.2 Hz, NH-CO), 7.10–7.82 (38H, m, aromatics).

*H-Cys(Trt)-Cys(Trt)-OtBu (**2**)*

To a solution of the above reported *tert*-butyl ester **1** (7.0 g, 7.1 mmol) in CH_2Cl_2 (50 mL) DBU (1.1 g, 7.1 mmol) was added at room temperature. After 15 min the solution was evaporated to dryness and the residue chromatographed on silica gel using $CHCl_3$:hexane (9:1) as eluant to give pure *N*-deprotected dipeptide ester **2** as an oil (4.9 g, 90%). – $[\alpha]$D + 18.0° (c 1, $CHCl_3$). – Rf (b) 0.31. – IR ($CHCl_3$): 3360 br, 1730, 1670–1650 cm^{-1}. – ^{1}H-NMR ($CDCl_3$) δ = 1.35 (9H, s, 3 × CH_3), 1.40 (2H, br, NH_2), 2.48 (2H, m, Cys β-CH_2), 2.53 and 2.78 (2H, m, Cys β-CH_2), 3.02 (1H, m, Cys α-CH), 4.41 (1H, m, Cys α-CH), 7.10–7.54 (30H, m, aromatics), 7.70 (1H, d, J = 7.0 Hz, NH).

*γ(Boc-Glu-OtBu)-Cys(Trt)-Cys(Trt)-OtBu (**3**)*

To a stirred ice-cold solution of Boc-Glu(OH)-O*t*Bu (1.8 g, 5.9 mmol) and the above reported dipeptide *tert*-butyl ester **2** (4.5 g, 5.9 mmol) in THF (30 mL) DCCI (1.2 g, 5.9 mmol) in THF (8 mL) was added portionwise. After 4 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the resulting solution evaporated under vacuum to give a residue which was taken up in EtOAc and worked up as described for compound **1**. Purification by silica gel column chromatography using $CHCl_3$:ether (96:4) as eluant afforded pure tripeptide di-*tert*-butyl ester **3** as a foam (5.2 g, 84%). – $[\alpha]$D +8.0° (c 1, $CHCl_3$). – Rf (c) 0.55. – IR ($CHCl_3$):3410, 3380, 1725, 1710, 1640, 1490 cm^{-1}. – ^{1}H-NMR ($CDCl_3$) δ = 1.42 (9H, s, 3 × CH_3), 1.45 (18H, s, 6 × CH_3), 1.70–1.98 (2H, m, Glu β-CH_2), 2.18 (2H, m, Glu γ-CH_2), 2.50 (2H, m, Cys β-CH_2), 2.58 and 2.78 (2H, m, Cys β-CH_2), 4.07 (1H, m, Cys α-CH), 4.22 (1H, m, Glu α-CH), 4.33 (1H, m, Cys α-CH), 5.23 (1H, d, J = 7.5 Hz, Glu NH), 6.22 (1H, d, J = 7.0 Hz, Cys NH), 6.67 (1H, d, J = 7.0 Hz, Cys NH), 7.13–7.52 (30H, m, aromatics).

γ(Boc-Glu-OtBu) -Cys-Cys- *OtBu (**4**)*

To a stirred solution of I_2 (3.0g, 11.9mmol) in MeOH (350mL) di-*tert*-butyl ester **3** (5.0g, 4.75mmol) in MeOH (100mL) was added portionwise at room temperature during 45min. After 4h under stirring the reaction mixture was cooled at 0°C and decoulorized with 1N $Na_2S_2O_3$. The residue obtained after removal of the solvent was partitioned between H_2O and EtOAc and the organic layer was washed with 1N $Na_2S_2O_3$ and H_2O. Drying and evaporation followed by purification by silica gel column chromatography ($CHCl_3$:MeOH 97:3 as eluant) of the resulting crude product afforded pure disulfide ester **4** (2.2g, 82%) as a foam. – $[\alpha]_D$ −28.0° (c 1, ($CHCl_3$). – Rf (d) 0.52. – IR ($CHCl_3$): 3410, 3380, 1730, 1710, 1660, 1500cm^{-1}. – ^{1}H-NMR ($CDCl_3$) δ = 1.45 (27H, s, 9 × CH_3), 1.85 (2H, m, Glu β-CH_2), 2.28 (2H, m, Glu γ-CH_2). 2.85–3.42 (4H, m, 2 × Cys β-CH_2), 4.18 (1H, m, Glu α-CH) 4.72–4.90 (2H, m, 2 × Cys α-CH), 5.23 (1H, d, J = 7.0Hz, Glu NH), 6.50 (1H, br d, Cys NH), 7.14 (1H, br d, Cys NH).

γ-(H-Glu-OH) -Cys-Cys- *OH (5)*

An ice-cooled solution of the protected disulfide **4** (2.0g, 3.55mmol) in EtOAc (18mL) was treated with dry HCl gas and was then stirred for 1h at room temperature. The hygroscopic solid which separated was collected and repeatedly washed with ether to give 1.3g (95%) of HCl · γ-(H-Glu-OH) -Cys-Cys- OH which was used without further purification.

The hydrochloride (1.3g, 3.4mmol) was dissolved in 1N aqueous NH_3 (50mL) at room tempetature. After 30min the aqueous solution was concentrated and subjected to column chromatography on Sephadex LH-20 using H_2O as eluent to afford disulfide **5** as white amophous solid (1.0g, 84%). – $[\alpha]_D$ −88.0° (c 1, H_2O). – Rf (e) 0.33. – Rf (f) 0.55. – Rf (g) 0.33. – IR (KBr) 3250br, 3190br, 1650–1550, 1400, 1230cm^{-1}. – ES-MS: 223.0 (Cys-Cys · H^+, 15%), 277.0 (15%), 352.0 (MH^+, 100%), 702.6 (2M + H^+, 85%) m/z. The MS/MS spectrum of the 2M + H^+ species shows only two peaks at 351.5 and 701.9m/z.

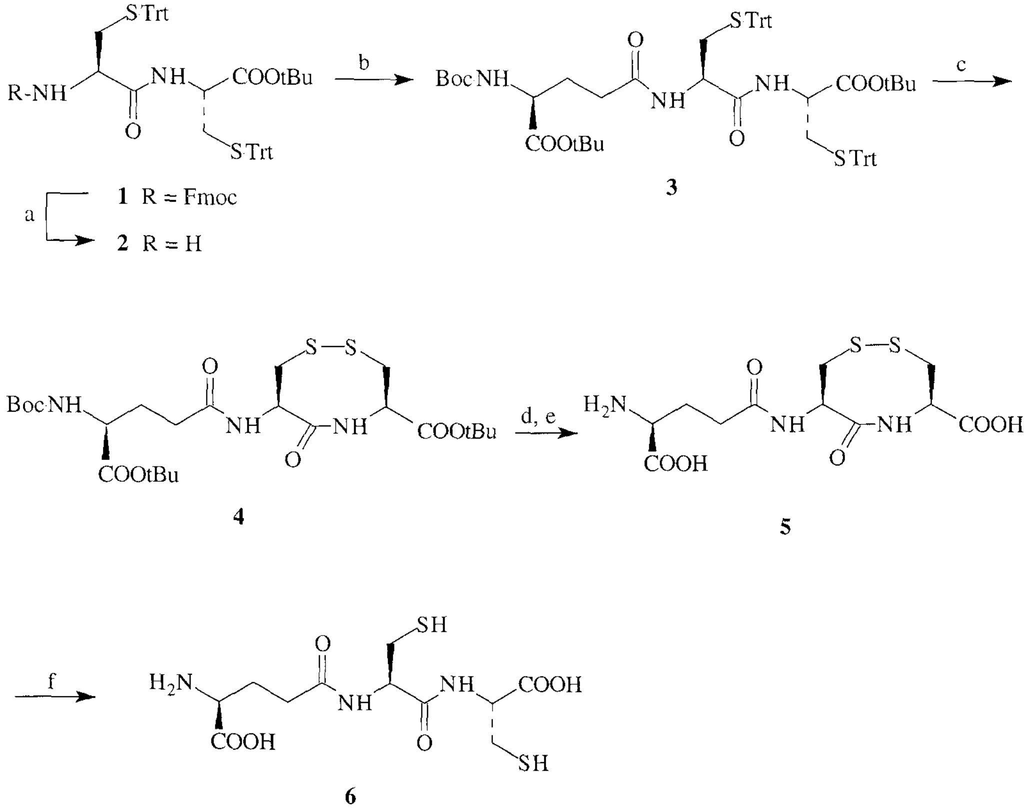
*γ-(H-Glu-OH)-Cys-Cys-OH (**6**)*

A solution of the above reported disulfide **5** (0.9g, 2.5mmol) in a mixture of *n*-PrOH : H_2O (2:1) (50mL) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. Tri-*n*-butylphosphine (0.6g, 3.0mmol) was added and the stoppered flask stirred at room temperature. After 1h the reaction mixture was repeatedly washed with $CHCl_3$ and the pH of the aqueous solution adjusted to 6.0 by 1N HCl. The solution was concentrated and subjected to column chromatography as described for disulfide **5** to give title compound **6** as a vitrous foam (0.8g, 90%). – $[\alpha]_D$ −18.0° (c 0.7, H_2O). – Rf (e) 0.37. – Rf (f) 0.58. – Rf (g) 0.31. – IR (KBr) 3280br, 2570, 1650–1540, 1380, 1245cm^{-1}.

Results

As useful strategy to the GSH analogue **5**, coupling of the γ-Glu residue with the preformed cyclic disulfide was first considered. Thus, Fmoc -Cys-Cys- O*t*Bu was synthesized as key intermediate; *N*-deprotection was first tried by using triethylamine in CH_2Cl_2 as mild deblocking agent (Chang et al., 1980). TLC monitoring of the reaction mixture revealed that the Fmoc cleavage was largely ineffective (more than 80% of the starting material after 24 hours at r.t.). When the stronger deblocking agent DBU was adopted, the deprotection was complete after 15min. at r.t.; the desired

H-Cys-Cys-O*t*Bu, however, was contaminated by impurities and degradation products requiring tedious purification steps. As an alternative route the incorporation of the γ-Glu residue onto the linear Cys-Cys fragment was considered (see Scheme 1). As in the case of previously reported GSH analogues (Calcagni et al., 1996), the protection of both the carboxy functions as *tert*-butyl esters, removable under acidic conditions, was necessary to assure satisfactory yields. Thus, the *S*-triphenylmethyl (*S*-Trt) derivative **3** was selected as suitable intermediate to **5** and obtained in 85% yield by coupling Boc-Glu(OH)-O*t*Bu with H-Cys(Trt)-Cys(Trt)-O*t*Bu **2**. Selective removal (Kamber, 1971) under high dilution conditions of the Trt-protecting groups, by following the Cumberbatch protocol (Cumberbatch et al., 1993), allowed direct conversion of **3** into the monomeric cyclic disulfide **4** (82% yield). Subsequent removal in a single step of all protecting groups, followed by treatment of the resulting hydrochloride with aqueous ammonia, gave the fully deprotected GSH analogue **5** in high yield. The dithiol form **6** can be obtained in 90% yield by reductive cleavage of the disulfide link of **5** with tri-



Scheme 1. Reagents and conditions: **a)** DBU, CH_2Cl_2, RT, 15 min; **b)** Boc-Glu(OH)-O*t*Bu, DCCI, THF, 0°C, 4 h, then 5°C, 16 h; **c)** I_2, MeOH, RT, 4 h; **d)** HCl, EtOAc, RT, 1 h; **e)** 1 N aq. ammonia, RT, 30 min; **f)** $(n\text{-Bu})_3P$, n-PrOH-H_2O (2 : 1), RT, 1 h

n-butylphosphine (1.2 equiv.) in water/*n*-propanol solution, by operating at pH 8.5 (aqueous ammonia) (Calcagni et al., 1995).

Peptide purity was assessed by HPLC and TLC. Under the conditions employed for HPLC analysis compounds **5** and **6** eluted as a single peak (retention time: 26.5 and 12.0 min. for **5** and **6**, respectively).

The structure assigned to the GSH analogues **5** and **6** is in accordance with the spectroscopic properties (see Table 1). The NMR spectra of compound **5** reveal that two main conformations are present in D_2O solution. In agreement with literature data (Brady et al., 1993; Liff et al., 1996; Horne et al., 1993) different helicity of the -S-S- bond inside a *cis*-amide containing 8-membered ring should represent the preferred conformations. In the ^1H-NMR spectra the αCys protons of the major conformer of peptide **5** (see Table 1) are downfield shifted (4.72 ppm) as compared with the corresponding protons of the dithiol peptide **6**, which appear in the usual range for *trans*-configurated Cys-Cys amide bond (*ca.* 4.4 ppm) (Liff et al, 1996). A characteristic feature (Theriault et al., 1984; Calcagni et al., 1996) of the ^{13}C-NMR spectrum of **6** is represented by the resonances of the two Cys β-carbon atoms (28.15 and 28.80 γ ppm) which are shifted at higher field as compared with the corresponding atoms in the disulfide precursor **5** (49.92 and 45.77 δ ppm). The electrospray mass spectrum (ES-MS) of **5** shows the formation of both $M + H^+$ and $2M + H^+$ species. The monomeric nature is proved by the MS/MS spectrum of the $2M + H^+$ species which shows, also at different collision energies, the only production of the $M + H^+$ ion. The absence of further collisionally-induced fragment ions indicates that the $2M + H^+$ ions are generated through a clustering reaction ($MH^+ + M \rightarrow 2M + H^+$) during

Table 1. ^1H- (300 MHz) and ^{13}C- (75.43 MHz) NMR data[a] for compounds **5**[b] and **6**

Residue	γ-(H-Glu-OH) -$\overline{\text{Cys-Cys}}$- OH **5**		γ-(H-Glu-OH) -Cys-Cys-OH **6**	
	γ_C	γ_H	γ_C	γ_H
Glu				
C^α	56.78[c]	3.62 t	56.88[c]	3.67 t
C^β	28.97	2.02 m	29.08	2.05 m
C^γ	34.06	2.23–2.52 m	34.20	2.42 m
COO	178.68[d]	–	176.62[d]	–
CONH	177.22[d]	–	177.67[d]	–
Cys				
C^α	56.58[c]	4.72 m	58.65[c]	4.32[f] m
C^β	49.92[e]	2.58–3.35 m	28.15[e]	2.83 m
CO	176.80[d]	–	174.25[d]	–
Cys				
C^α	56.98[c]	4.72 m	59.20[c]	4.47[f] m
C^β	45.77[e]	2.58–3.35 m	28.80[e]	2.83 m
CO	178.93[d]	–	178.08[d]	–

[a] In D_2O at 23°C. Chemical shifts are relative to internal dioxane. [b] Data refer to the prevalent conformer. [c,d,e,f] Assignments may be interchanged.

the ionization phenomenon and are not due to the dimeric nature of the sample.

Studies on the conformational properties and the biological role that this new GSH analogue, containing the disulfide bridge as molecular constraint, may play on enzymes that control the GSH metabolism are in progress in our laboratories.

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