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Synthesis and biological evaluation of the disulfide form of the glutathione analogue γ-(L-glutamyl)-L-cysteinyl-L-aspartyl-L-cysteine

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Abstract

By using the chain to chain mode of cyclization the title glutathione analogue γGlu-Cys-Asp-Cys (**4**), containing the 11-membered disulfide ring -Cys-Asp-Cys- replacing the native -Cys-Gly fragment, has been synthesized and characterized together with its reduced dithiol form γ-Glu-Cys-Asp-Cys (**5**). The activity of (**4**) with γ-glutamyl-transferase and glutathione reductase has been evaluated and compared with those of the two conformationally restricted glutathione analogues (**2**) and (**3**) previously reported.

Keywords: Amino acids; Conformational constraint; Cyclic disulfides; Cyclotripeptides; Dithiols; Glutathione analogues

1. Introduction

Disulfide bridges represent important evolutionarily conserved structural motifs in many natural peptides and proteins, including hormones, enzymes, growth factors, toxins, and immunoglobulins [1]. In recent years intensive efforts have been

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made to develop mimetics of bioactive natural peptides containing cyclic disulfide frameworks in order to obtain analogs with better therapeutical potential than the endogenous prototypes. This strategy provides a useful way to stabilize specific backbone conformations and limit the rotational space of the side chains [2,3].

In the context of our studies on glutathione (GSH, **1**)[1] analogues [4–6] we recently adopted this approach by synthesizing the two disulfide analogs **2** and **3** characterized by the presence of the 8-membered disulfide ring -Cys-Cys- and the achiral and $C^{\alpha,\alpha}$tetrasubstituted 4-amino-1,2-dithiolane-4-carboxylic acid, respectively [7,8]. These modifications introduce new reactivities such as the participation in redox equilibria involving intra- or intermolecular disulfide forms [9–11], and have biological and conformational consequences resulting from the stabilization of specific backbone conformations and the restriction of the freely rotating side-chain of cysteine.

As part of our study on conformationally restricted GSH analogues we report here the synthesis of a new tetrapeptide model in which the backbone constraint is obtained by the incorporation of a cyclic fragment, namely -Cys-Asp-Cys- which replaces the native C-terminal -Cys-Gly- dipeptide. In the resulting GSH analogue γGlu-Cys-Asp-Cys **4**, the presence of the C-terminal carboxyl group, which is a prerequisite for the GSH activity, is maintained due to the presence of the aspartic acid residue.

In contrast with previously reported cyclic GSH analogues, in which the residue of the glutamic acid is part of the ring system [12], in **4** the γ-glutamyl moiety is located outside the ring and is not directly involved in the conformational constraint. Furthermore, due to the presence of the two adjacent cysteine residues, the tetrapeptide molecule could participate in intramolecular or intermolecular dithiol–disulfide interchange reactions.

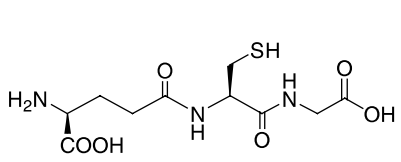
Although several 11-membered disulfide cyclic tripeptide -Cys-Xaa-Cys systems have been synthesized and studied [13–16], the tetrapeptide **4** is, to the best of our knowledge, the first synthetic γ-glutamyl derivative of an 11-membered disulfide cyclotripeptide to be reported. It should be also noted that a γ-glutamyl derivative analogous to **4**, namely γGlu-Cys-Gly-Cys has been detected among the neuropeptides present in the bovine brain [17].

Data concerning the activity of **4** with two glutathione dependent enzymes, γ-glutamyltranspeptidase (γ-GT, EC 2.3.2.2) and glutathione reductase (GR, EC 1.6.4.2), are reported and compared with that of analogues **2** and **3**. Interest in γ-GT is due to the metabolic stability of GSH and its analogues [18] as well as in its expression and function in carcinogenesis [19]. The GR, on the other hand, is a flavoprotein that plays a key antioxidant role through its intracellular function of maintaining a high GSH–GSSG ratio [20]. GSH analogues able to interfere with its functions may assist in obtaining information about its role in the development of oxidative stress [21].

[1] *Abbreviations used:* Abbreviations follow the recommendations of the IUPAC-IUB Commision on Biochemical Nomenclature as given in Eur. J. Biochem. 138 (1984) 9–37. Additional abbreviations: DBU, 1,8-diazabicyclo 5.4.0 undec-7-ene; DCCI, *N*,*N*-dicyclohexylcarbodiimide; GPNA, γ-glutamyl-*p*-nitroanilide; HETCOR, heteronuclear chemical shift correlation experiment; NADPH, β-nicotinamide adenine dinucleotide phosphate reduced; NMM, *N*-methylmorpholine; THF, tetrahydrofuran; Trt, triphenylmethyl; $(n\text{-Bu})_3$P, tri-*n*-butylphosphine; GSH, glutathione reduced form; GSSG, glutathione oxidized; γ-GT, γ-glutamyltranspeptidase; GR, glutathione reductase.

2. Chemistry

The first synthetic strategy adopted to prepare the GSH analogue **4** was the incorporation of the γ-Glu residue onto the preformed 11-membered cyclic disulfide. Accordingly, Fmoc-Cys-Asp(OtBu)-Cys-OtBu was synthesized as suitable intermediate. However, as was already found in the case of the 8-membered cyclic disulfide Fmoc-Cys-Cys-OtBu [7], the *N*-deprotection represented a critical step and notwithstanding the use of different deblocking agents, the desired Cys-Asp-(OtBu)-Cys-OtBu was only obtained in low yield and was contaminated by impurities and degradation products. As an alternative route to **4**, coupling of the γ-Glu residue with the linear Cys-Asp-Cys fragment was considered (Scheme 1). In order to achieve satisfactory yields, the use of the *tert*-butyl esters, removable under acidic conditions, was adopted [6]. Thus (see Scheme 1) the key intermediate tetrapeptide γ(Boc-Glu-OtBu)-Cys(Trt)-Asp(OtBu)-Cys(Trt)-OtBu **11** was synthesized by a stepwise elongation using the *N*,*N*-dicyclohexylcarbodiimide (DCCI) method. For the *N*-deprotection of the Fmoc-intermediates **7** and **9**, 1,8-diazabicyclo 5.4.0 undec-7-ene (DBU) in CH_2Cl_2 was chosen [6]. Selective removal under mild conditions (I_2 in MeOH, room temperature) of the Trt protecting groups under high dilution conditions [7] allowed direct conversion of **11** to the N-Boc cyclic [1,3] disulfide **12**. It should be noted that the yield (35%) of this deprotection-cyclization step is significantly lower than that previously reported by us for the 8-membered cyclic disulfide **2**. In the present case a more pronounced tendency to form oligomeric products was observed and this led to the adoption of higher dilution conditions (10^{-3} M vs 10^{-2} M ca.). Subsequent removal in a single step of all protecting groups, followed by treatment of the resulting hydrochloride with aqueous ammonia, afforded the GSH analogue **4** in good yields. Reductive cleavage of the disulfide link of **4** with tri-*n*-butyl phosphine in water/*n*-propanol solution [5] gave the dithiol form **5** in 70% yield. The purity of **4** was determined by HPLC and TLC. Under the conditions adopted for HPLC analysis, compound **4** eluted as a single peak (retention time: 17.7 min).



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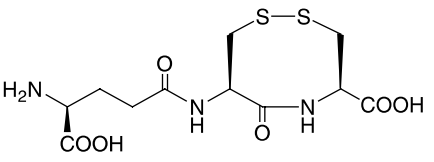

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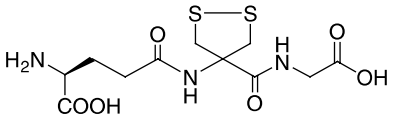

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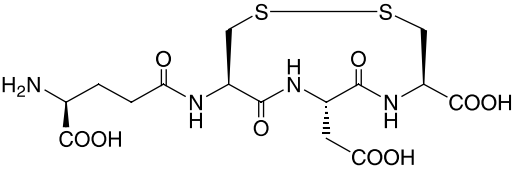

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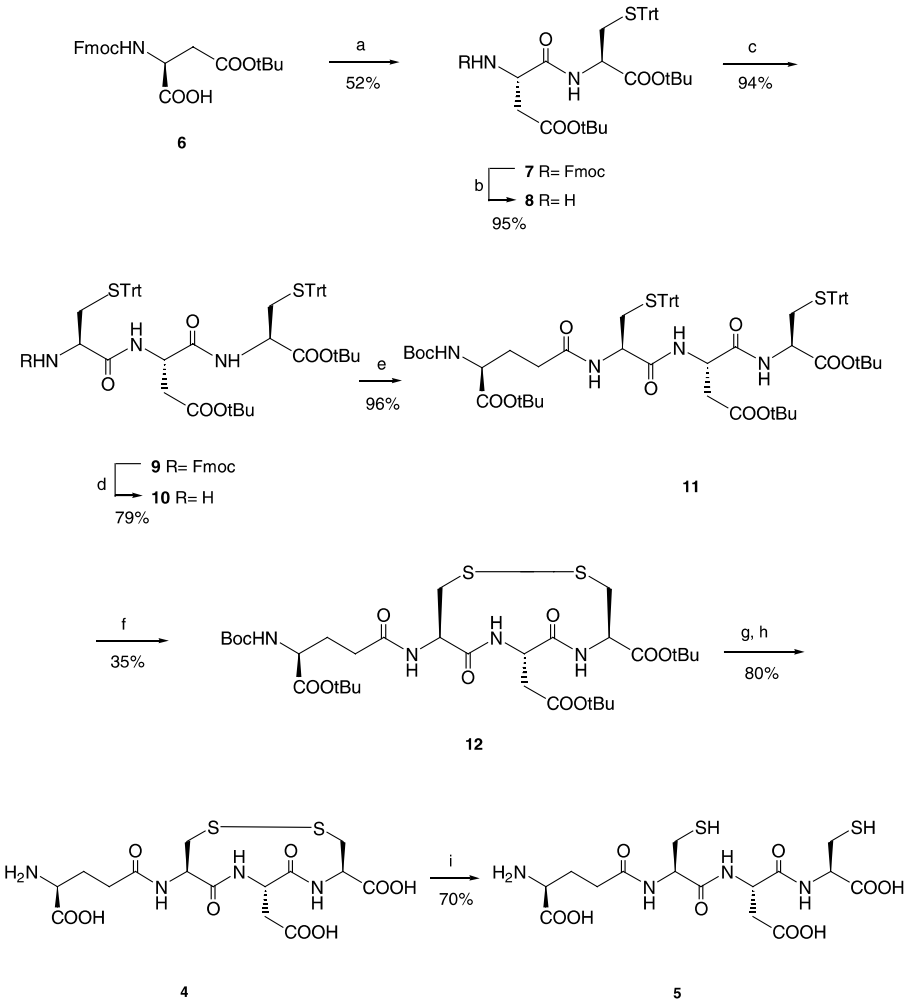

Scheme 1. Reagents and conditions. (a) Cys(Trt)OtBu, DCCI, THF 0 °C, then 5 °C, 16 h; (b) DBU, CH_2Cl_2, rt; 15 min; (c) Fmoc-Cys(Trt)-OH, DCCI, THF, 0 °C, 4 h, then 5 °C, 16 h; (d) DBU, CH_2Cl_2, rt, 15 min; (e) Boc-Glu-OtBu, DCCI, THF, 0 °C, 4 h, then 5 °C, 16 h; (f) I_2, MeOH, rt, 4 h; (g) HCl, ethyl acetate, rt, 1 h; (h) 1 N aq. NH_3, rt, 30 min; (i) $(n\text{-}Bu)_3P$, n-PrOH-H_2O (2:1), rt, 1 h.

The structure assigned to the GSH analogues **4** and **5** is in accordance with the spectroscopic properties (see Table 1). In particular, the NMR spectra of the linear dithiol form **5** reveal a single set of resonances. On the contrary, the spectral data of the cyclic disulfide **4** show the existence of more than one set of signals, thus indicating the presence (under the conditions of the NMR experiment) of slowly interconverting multiple conformational states. In particular, analogue **4** showed three complete sets of signals (relative intensity 1:0.7:0.3) which, on the basis of accurate HPLC analysis, can be attributed to different conformers. A characteristic feature

Table 1
^{1}H- and ^{13}C-NMR parameters[a] of compounds **4**[b] and **5**

Residue	γGlu-Cys-Asp-Cys (**4**)		γ-Glu-Cys-Asp-Cys (**5**)	
	δ_H	δ_C	δ_H	δ_C
Glu				
C^{α}	3.59 t (5.12)	54.26	3.59 t (5.15)	54.25
Cβ	1.90–2.12 m	26.35	1.90–2.05 m	30.17
C^{γ}	2.24–2.50 m	31.53	2.30–2.41 m	37.90
COO	–	176.70[c]		172.47[d]
CONH	–	177.67[c]		176.04[d]
Cys				
C^{α}	4.45–4.54 m	53.42	4.16–4.22 m	56.68
C^{β}	2.75–2.90 m	38.86	2.70–2.90 m	26.39
CO	–	174.79[c]		175.03[d]
Asp				
C^{α}	4.30–4.42 m	51.21	4.51–4.59 m	51.60
C^{β}	2.50–2.70 m	37.62	2.45–2.70 m	31.45
CO	–	171.85[c]		172.32[d]
Cys				
C^{α}	4.45–4.55 m	52.44	4.31–4.40 m	55.90
C^{β}	3.00–3.20 m	38.06	2.70–2.90 m	26.28
CO	–	174.16[c]		174.19[d]

[a] In D_2O at 25 °C. *J*/Hz in parentheses.
[b] Data refer to the predominant conformer.
[c] Assignments may be interchanged.
[d] Assignments may be interchanged.

of the ^{13}C-NMR spectrum of **4** is represented by the resonances of the two Cys β-carbon atoms (38.06 and 38.86 ppm) which are downfield shifted compared with the corresponding atoms in the dithiol derivative **5** (26.28 and 26.39 ppm). This spectral behaviour represents, as recently noted [22], a reliable diagnostic feature for distinguishing between cysteine and cystine containing forms. Mass spectra of **4** and **5** show the most abundant peaks correspond to $M + H^+$ species (467 and 469 m/z, respectively). Furthermore, high resolution analysis of the respective molecular ion region demonstates the monomeric nature of **4** and **5**.

3. Materials and methods

TLC was performed on Merck 60 F_{254} silica gel plates. HPLC was performed with a Waters system equipped with a M-600 multisolvent system and a 2487 dual waves UV detector linked to a Millenium 32 data station using a Merck C-18 reversed-phase column (125 × 4 mm) "Purospher" with 3 μm packing material. The solvent used was 0.1% aqueous trifluoroacetic acid/methanol 80/10 at the flow rate of 1.0 ml/min. The eluant was detected at 210 nm. Column chromatography was carried out using Merck 60 silica gel (230–400 mesh). Optical rotations were obtained at

25 °C with Perkin–Elmer 241 polarimeter. IR spectra were recorded employing a Perkin–Elmer 983 FTIR 1600 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were determined on a Varian VXR 300-MHz spectrometer (δ expressed in ppm). The assigment of ^{1}H- and ^{13}C-NMR resonances of analogues **4** and **5** (Table 1) was achieved with standard 2D NMR spectroscopic techniques (HETCOR). The mass spectra of **4** and **5** have been obtained in electrospray (ES) conditions by a LCQ (Finnigan, San. Jose, CA, USA) instrument. The 60 μM solution of the sample in CH_3OH:H_2O (50:50) containing 1% acetic acid was directly injected into the instrument at the flow rate of 5 μl/min.

Cys(Trt)-OtBu, Fmoc-Cys(Trt)-OH, Fmoc-Asp(OtBu)-OH and Boc-Glu-OtBu were obtained from Novabiochem (Läufelfingen, Switzerland, EU).

3.1. Fmoc-Asp(OtBu)-Cys(Trt)-OtBu (7)

DCCI (3.6 g, 17.5 mmol) was added portionwise at 0 °C to a stirred solution of Fmoc-Asp(OtBu)-OH **6** (7.2 g, 17.5 mmol) and Cys(Trt)-OtBu (7.4 g, 17.5 mmol) in THF (30 ml). After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in ethyl acetate and the organic layer washed with 1 M $KHSO_4$, saturated aqueous Na_2CO_3 and H_2O. The residue obtained after drying and evaporation was chromatographed on silica gel using cyclohexane/diethyl ether 1:1 as eluant to yield dipeptide di-*tert*-butyl ester **7** as a white foam (7.4 g, 52%). $[\alpha]_D = +20.0°$ ($c = 1$, $CHCl_3$). $R_f = 0.42$ (cyclohexane/diethyl ether, 1:1). IR (nujol): 3310, 1725, 1670, 1490 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.45 (18H s, 6× CH_3), 2.58 (2H, m, Cys β-CH_2), 2.77 (2H, d, Asp β-CH_2), 4.15–4.45 (3H, m, Fmoc CH–CH_2), 4.40 (1H, m, Cys α-CH), 4.60 (1H, m, Asp α-CH), 6.01 (1H, d, $J = 8.25$ Hz, Asp NH), 7.13 (1H, d, $J = 7.70$ Hz, Cys NH), 7.10–7.82 (23H, m, aromatic protons).

3.2. Asp(OtBu)-Cys(Trt)-OtBu (8)

DBU (1.4 g, 8.6 mmol) was added at room temperature to a solution of the di-*tert*-butyl ester **7** (7.0 g, 8.6 mmol) in CH_2Cl_2 (65 ml). After 20 min the solution was evaporated to dryness and the residue chromatographed on silica gel using $CHCl_3$/MeOH 99:1 as eluant to give pure *N*-deprotected dipeptide ester **8** as a yellow oil (4.8 g, 95%). $[\alpha]_D = +11.0°$ ($c = 1$, $CHCl_3$). $R_f = 0.42$ ($CHCl_3$/MeOH, 99:1). IR (nujol): 3370 (brd), 1725, 1675, 1505 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.45 (18H, s, 6×CH_3), 1.80 (2H, s, NH_2), 2.51 (2H, m, Cys β-CH_2), 2.70 (2H, m, Asp β-CH_2), 3.65 (1H, m, Asp α-CH), 4.42 (1H, m, Cys α-CH), 7.10–7.51 (15H, m, aromatic protons), 7.84 (1H, d, $J = 8.10$ Hz, Asp NH).

3.3. Fmoc-Cys(Trt)-Asp(OtBu)-Cys(Trt)-OtBu (9)

DCCI (1.6 g, 7.6 mmol) was added portionwise at 0 °C to a stirred solution of Fmoc-Cys(Trt)-OH (4.45 g, 7.6 mmol) and the di-*tert*-butyl ester of the dipeptide **8** (4.5 g, 7.6 mmol) in THF (18 ml). After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture

was filtered and the resulting solution was evaporated under vacuum to give a residue which was taken up in ethyl acetate and worked up as described for compound **7**. Purification by silica gel column chromatography using cyclohexane/diethyl ether 1:1 as eluant afforded pure tripeptide di-*tert*-butyl ester **9** as a white foam (8.3 g, 94%). $[\alpha]_D = +18.0°$ ($c = 1$, $CHCl_3$). $R_f = 0.29$ (cyclohexane/diethyl ether, 1:1). IR (nujol): 3320 (brd), 1725, 1675, 1505 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.42 (18H, s, 6× CH_3), 2.41 and 2.71 (4H, m, 2× Cys β-CH_2), 2.78 (2H, m, Asp β-CH_2), 3.65 (1H, m, Cys α-CH), 4.15–4.45 (3H, m, Fmoc CH–CH_2), 4.18 (1H, m, Cys α-CH), 4.63 (1H, m, Asp α-CH), 4.90 (1H, d, $J = 7.05$ Hz, Cys NH), 7.03 (1H, d, $J = 7.91$ Hz, Asp NH), 7.20 (1H, obs, Cys NH), 7.15–7.81 (38H, m, aromatic protons).

3.4. *Cys(Trt)-Asp(OtBu)-Cys(Trt)-OtBu* (**10**)

DBU (1.1 g, 6.9 mmol) was added at room temperature to a solution of the tripeptide di-*tert*-butyl ester **9** (8.0 g, 6.9 mmol) in CH_2Cl_2 (53 ml). After 20 min the solution was evaporated to dryness and the residue chromatographed on silica gel using $CHCl_3$/MeOH 99:1 as eluant to give pure *N*-deprotected tripeptide ester **10** as a white foam (5.1 g, 79%). $[\alpha]_D = +10.5°$ ($c = 1$, $CHCl_3$). $R_f = 0.36$ ($CHCl_3$/MeOH 99:1). IR (nujol): 3345 (brd), 1725, 1675, 1500 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.49 (18H, s, 6× CH_3), 1.51 (2H, br, NH_2), 2.45–2.75 (6H, m, 2× Cys β-CH_2 and Asp β-CH_2), 2.96 (1H, m, Cys α-CH), 4.30 (1H, m, Cys α-CH), 4.69 (1H, m, Asp α-CH), 7.09 (1H, d, $J = 6.01$ Hz, Cys NH), 7.11–7.50 (30H, m, aromatic protons), 8.11 (1H, d, $J = 7.72$ Hz, Asp NH).

3.5. *γ(Boc-Glu-OtBu)-Cys(Trt)-Asp(OtBu)-Cys(Trt)-OtBu* (**11**)

DCCI (1.1 g, 5.3 mmol) was added portionwise at 0 °C to a stirred ice-cold solution of Boc-Glu-OtBu (1.6 g, 5.3 mmol) and the tripeptide di-*tert*-butyl ester **10** (5 g, 5.3 mmol) in THF (30 ml). After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum to give a residue which was taken up in ethyl acetate and worked up as described for compound **7**. Purification by silica gel column chromatography using $CHCl_3$/diethyl ether 1:1 as eluant afforded pure tetrapeptide tri-*tert*-butyl ester **11** as a white foam (6.2 g, 96%). $[\alpha]_D = +11.5°$ ($c = 1$, $CHCl_3$). $R_f = 0.68$ ($CHCl_3$/diethyl ether, 1:1). IR (nujol): 3405 (brd), 1725, 1665, 1495 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.42 (9H, s, 3× CH_3), 1.45 (27H s, 9× CH_3), 1.81 (2H, m, Glu β-CH_2), 2.21 (2H, m, Glu γ-CH_2), 2.30–2.70 (4H, m, 2× Cys β-CH_2), 2.70 (2H, m, Asp β-CH_2), 3.85 (1H, m, Cys α-CH), 4.17 (2H, m, Cys α-CH and Glu α-CH), 4.70 (1H, m, Asp α-CH), 5.22 (1H, d, $J = 8.24$ Hz, Glu NH), 7.05 (1H, d, $J = 7.90$ Asp NH), 7.10 (1H, $J = 6.00$, Cys NH), 7.21–7.59 (30H, m, aromatic protons), 7.40 (1H, obs, Cys NH).

3.6. *γ(Boc-Glu-OtBu)-Cys-Asp(OtBu)-Cys-OtBu* (**12**)

The tetrapeptide tri-*tert*-butyl ester **11** (6.0 g, 4.9 mmol) in MeOH (1500 ml) was added portionwise at room temperature over 1 h to a stirred solution of I_2 (2.5 g,

9.8 mmol) in MeOH (3500 ml). After 4 h under stirring the reaction mixture was cooled at 0 °C and decolorized with 1 M $Na_2S_2O_3$. The residue obtained after removal of the solvent was partitioned between H_2O and ethyl acetate and the organic layer was washed with 1 M $Na_2S_2O_3$ and H_2O. Drying and evaporation followed by purification by silica gel column chromatography ($CHCl_3$/*i* Pr-OH 97:3 as eluant) of the resulting crude product afforded pure disulfide ester **12** (1.2 g, 35%). $[\alpha]_D = -22.0°$ ($c = 1$, $CHCl_3$). $R_f = 0.35$ ($CHCl_3$/*i* Pr-OH, 97:3). IR ($CHCl_3$): 3415 (brd), 1725, 1690, 1505 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.45 (36H s, 12× CH_3), 1.83 (2H, m, Glu β-CH_2), 2.30 (2H, m, Glu γ-CH_2), 2.76 (2H, m, Cys β-CH_2), 2.91–3.29 (4H, m, Cys β-CH_2 and Asp β-CH_2), 4.13 (1H, m, Glu α-CH), 4.54 (1H, m, Cys α-CH), 4.61–4.89 (2H, m, Cys α-CH and Asp α-CH), 5.25 (1H, d, $J = 7.92$ Hz, Glu NH), 6.86 (1H, d, $J = 7.03$ Hz, Cys NH), 7.40 (1H, d, $J = 6.32$ Hz, Cys NH), 7.94 (1H, d, $J = 8.8$ Hz, Asp NH).

3.7. γGlu-$\overline{\text{Cys-Asp-Cys}}$ (*4*)

An ice-cooled solution of the protected disulfide **12** (1.2 g, 1.6 mmol) in EtOAc (11 ml) was treated with dry HCl gas and was then stirred for 1 h at room temperature. The igroscopic solid which separated was collected and repeatedly washed with diethyl ether to give 0.7 g of HCl·γGlu-$\overline{\text{Cys-Asp-Cys}}$ which was used without further purification.

The hydrochloride (0.7 g, 1.4 mmol) was dissolved in 1 M aqueous NH_3 (20 ml) at room temperature. After 30 min the aqueous solution was concentrated and subjected to column chromatography on Sephadex LH-20 using H_2O as eluent to afford disulfide **4** as a white amorphous solid (0.5 g, 80%). $[\alpha]_D = -47.0°$ ($c = 1$, H_2O). $R_f = 0.23$ (*n*-BuOH/acetic acid/H_2O, 4:5:1). IR (KBr): 3415 (brd), 1725, 1665, 1530, 1410, 1225 cm^{-1}. MS $M + H^+ = 467$ m/z.

3.8. γ-Glu-Cys-Asp-Cys (*5*)

A solution of the above reported disulfide **4** (0.5 g, 1.0 mmol) in a mixture of *n*-PrOH/H_2O (2:1) (24 ml) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. Tri-*n*-butyl phosphine (0.3 g, 1.5 mmol) was added and the stoppered flask was stirred at room temperature. After 1 h the reaction mixture was repeatedly washed with $CHCl_3$ and the pH of the aqueous solution adjusted to 6.0 using 1 M HCl. The solution was concentrated and subjected to column chromatography on Sephadex using H_2O/MeOH 2:1 as eluant to afford compound **5** as a vitrous foam (0.33 g, 70%). $[\alpha]_D = -56.0°$ ($c = 1$, H_2O). $R_f = 0.38$ (*n*-BuOH/acetic acid/H_2O, 4:5:1). IR (KBr): 3430 (brd), 3265, 1650, 1520, 1385, 1120 cm^{-1}. MS $M + H^+ = 469$ m/z.

4. Biochemistry

γ-GT from hog kidney (about 3 U/mg) and from bovine kidney (250 U/mg) were supplied by Fluka and Sigma, respectively. GR from bakers yeast (type III, 260 U/

mg) and from wheat germ (type II, 0.08 U/mg) were obtained from Sigma. GPNA, Gly–Gly, GSSG, GSH, NADPH were purchased from Sigma. In vitro assays of γ-GT and GR activity were performed according to Meister et al. [23] and Carlberg et al. [24], respectively.

5. Results and discussion

In order to test the putative biological activity of glutathione analogues **2–4**, inhibition studies have been performed on two enzymes: γ-GT and GR. The first enzyme catalyzes the transfer of the γ-glutamyl moiety from many donor molecules including GSH [25] to a large number of acceptor substrates [26,27]. The second enzyme is a flavoprotein catalyzing the NADPH-dependent reduction of glutathione disulfide (GSSG). The reaction is essential for maintenance of glutathione levels [24].

*5.1. GSH analogues **2–4** as γ-GT inhibitors*

The inhibition of the γ-glutamyl binding site of this enzyme has been reported previously [5,6]. According to Meister et al. [23] the reaction on the donor site (γ-glutamyl binding) was performed using pseudo-first-order conditions, with a saturating concentration of the acceptor molecule ([Gly–Gly] = 150 mM). Table 2 shows the residual enzymatic activity in the presence of specific inhibitors at three different concentrations. The two different enzyme batches did not show any differences in activity. Similar results were obtained with inhibitor **2** using a non-saturating concentration of the acceptor molecule ([Gly–Gly] = 15 mM), which is the concentration usually reported for enzyme activity determinations [23]. Compound **2** is more active than any of the other compounds tested.

Preincubation of the enzyme with inhibitors at 37 °C shows an effect only for compound **2** (Fig. 1). The observed improved inhibition with this compound, which depends from the preincubation time, excludes the possibility that it is acted on as substrate. A double reciprocal plot of inhibition data for compound **2** shows a non-competitive inhibition pattern at the γ-glutamyl site (data not

Table 2
Residual activities of γ-GT in the presence of different glutathione analogues **2–4**[a]

Compound	Inhibitor concentration		
	1 mM (%)	1.4 mM (%)	2.8 mM (%)
2	79	68	62
3	90	–	85
4	98	98	95[b]

[a] Enzyme concentration was about 50 mU/ml. GPNA as substrate and the inhibitor were added simultaneously to prime the reaction. Data are averages of at least three determinations. The experiments were carried out in 0.1 M Tris–HCl buffer, pH 8.0, 23 °C.

[b] At 6 mM concentration of **4**, a residual activity of 85% is obtained.

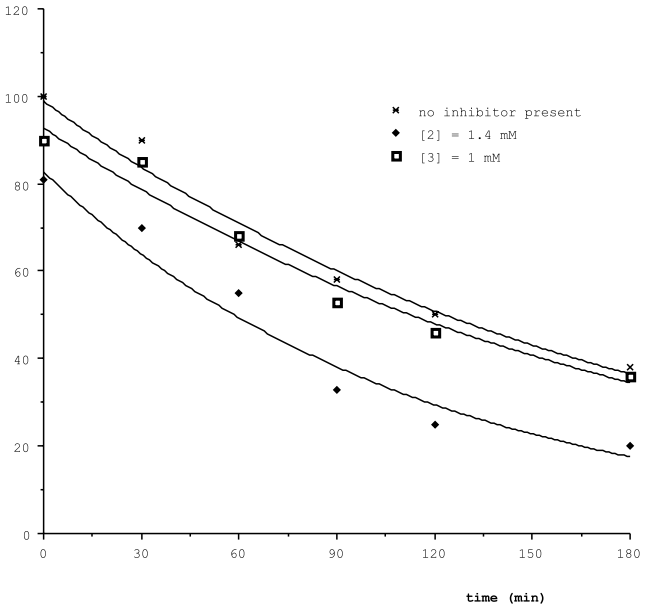

Fig. 1. Plot of residual enzymatic activity of γ-GT in the presence of two different inhibitors. Enzyme (about 50 mU/ml) was preincubated in 0.1 M Tris–HCl buffer, pH 8.0 at 37 °C for the given time, in the presence of *: no inhibitor present, ◆: [2] = 1.4 mM, ■: [3] = 1 mM. The activity was then measured by adding substrates [GPNA] = 1 mM and [Gly–Gly] = 150 mM.

shown) with an approximate K_i value of 0.7 mM. An analogous non-competitive inhibition was reported also for the γ-(L-γ-oxaglutamyl) analogue of GSH [6], whereas GSH and the γ-aza analogue exhibited competitive patterns [5]. These data suggest different interactions with the γ-glutamyl site of the enzyme by these glutathione analogues.

Compound **3** was also tested as substrate of γ-GT. After incubation at 37 °C for 1 h in the presence of the enzyme, the appearance of glutamic acid was monitored by amino acid analysis. The activity of the enzyme using compound **3** was about 2% of that using GPNA or using the natural substrate GSH [28]. Similar results were obtained using compound **4**.

*5.2. GSH analogues **2–4** as GR inhibitors*

The above glutathione analogues **2–4** were tested also using GR. Both enzyme batches were tested for inhibition. The experimental data (Table 3) show the residual activity of enzyme for three different concentrations of inhibitors. Very little inhibition was observed. Preincubation of the tested compounds with the enzyme for up to 60 min has no additional effect on activity. Compound **4** was also tested as putative substrate with NADPH. The reaction followed at 340 nm against suitable blank showed that the disulfide bond is not reduced by GR.

Table 3
Residual activities of GR in the presence of different glutathione analogues **2**–**4**[a]

Compound	Inhibitor concentration		
	0.5 mM (%)	1.0 mM (%)	2.0 mM (%)
2	93	85	90
3	83	73	NT
4	NT[b]	85	NT

[a] Enzyme concentration was about 50 mU/ml. The compound to be tested, [GSSG] = 1 mM and [NADPH] = 0.1 mM are added in the order and the reaction is followed at 340 nm. Data are averages of at least three determinations. The experiments were carried out at three concentrations, in 0.2 M K^+-phosphate buffer, pH 7.0, 23 °C.

[b] NT, not tested.

6. Conclusion

We report here the synthesis of novel glutathione analogues in which the typical γ-glutamyl residue is bound to a Cys-Asp-Cys tripeptide fragment. Both forms of the resulting tetrapeptide, i.e., the cyclic 11-membered disulfide **4** and the linear reduced dithiol **5**, have been isolated and fully characterized. Interest in these new type of glutathione analogues is due to the presence of the two cysteine residues within a single molecule which in the natural glutathione are responsible of the dithiol–disulfide oxidation and reduction as well as its antioxidant and scavenger properties. As a consequence of this structural feature and in contrast to what happens in the GSH–GSSG system, the disulfide bond reduction and oxidation may be involved in a unimolecular instead of a bimolecular reaction.

The biological activity of the cyclic disulfide analogues **2**–**4** has been tested with γ-GT and GR. A wide variety of γ-glutamyl derivatives has been in fact tested with these enzymes and an effective interaction has been shown with compounds in which the moieties attached to the γ-glutamyl group have considerably different structures [27]. By taking into account the structural analogy of **3** with the native GSH, we can reasonably argue that the low activity of this analogue is due to an unfavourable alteration of the backbone conformation involving the replacement of the native cysteine with the $C^{\alpha,\alpha}$tetrasubstituted 4-amino-1,2-dithiolane-4-carboxylic acid residue. α-Amino acid residues possessing a highly substituted α-carbon atom, here a spiro ring system, have restricted conformational freedom. This in turn leads to a more specific secondary structure capable of strongly influencing both the recognition and binding of macromolecular targets [29,30]. In the case of the two cyclic disulfide compounds **2** and **4**, containing the 8- and the 11-membered ring, respectively, the results clearly show that large ring structures with the γ-glutamyl residue do not favour interactions with the tested enzymes; only the 8-membered compound **2** shows in fact a weak inhibitory activity on γ-GT. Thus, in the case of compounds **2** and **4**, in contrast with **3**, the impairment in the accessibility to the active site due to steric reasons seems to be more likely.

As shown in Table 3, GR is not able to reduce the disulfide bond of the analogues nor is it strongly inhibited by these compounds. Because it is well known that GR prefers open chain dimeric or trimeric substrates [31,32], we can reasonably argue that the lack of activity of disulfide analogs **2**–**4** is due to their monomeric nature which is not compatible with binding at the GR active site.

A final consideration concerns the structure of the GSH analogues **4** and **5**. It is interesting to note that a strategy of structural modification of the GSH molecule analogous to that which led to **4** and **5**, is used by several pathogenic protozoa of the Trypanosoma and Leishmania genera.These protozoa do not depend on the GSH–GSSG system for their thiol redox balance and antioxidant defense. Instead they utilize a specific GSH derivative, N^1,N^8-diglutathionyl-spermidine, or trypanothione, in which the two Cys residues remain covalently bound through a spacer fragment, both in the reduced and oxidized form, as is the case for **4** and **5** [33]. Thus, the synthesis of the tetrapeptides **4** and **5** represents a preliminary step towards a new type of GSH–GSSG analogue which possess significant structural analogy with the redox system that is operative in several pathogenic microorganisms and may assist in the development of therapeutic strategies.

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