Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 1677–1683

BIOORGANIC & MEDICINAL CHEMISTRY

Proline–Glutamate Chimeras in Isopeptides. Synthesis and Biological Evaluation of Conformationally Restricted Glutathione Analogues

Mario Paglialunga Paradisi,[a] Adriano Mollica,[a] Ivana Cacciatore,[b] Antonio Di Stefano,[b] Francesco Pinnen,[b] Anna Maria Caccuri,[c] Giorgio Ricci,[c] Silvestro Duprè,[d] Alessandra Spirito[d] and Gino Lucente[a,*]

[a]*Istituto di Chimica Biomolecolare, CNR Sezione di Roma, c/o Dipartimento di Studi Farmaceutici, Università di Roma 'La Sapienza', P.le A. Moro, 00185 Rome, Italy*

[b]*Dipartimento di Scienze del Farmaco, Università 'G. D'Annunzio', Via dei Vestini 31, 66100 Chieti, Italy*

[c]*Dipartimento di Biologia, Università 'Tor Vergata', Via della Ricerca Scientifica, 00133 Rome, Italy*

[d]*Dipartimento di Scienze Biologiche, Università di Roma 'La Sapienza', P.le A. Moro, 00185 Rome, Italy*

Received 22 October 2002; accepted 3 January 2003

Abstract—The two novel diastereoisomeric glutathione analogues **1** and **2** have been designed and synthesized by replacing the native γ-glutamylic moiety with the conformational rigid skeleton of *cis*- or *trans*-4-carboxy-L-proline residue. Both analogues have been obtained by following the solution phase peptide chemistry methodologies and final reduction of the corresponding disulfide forms **13** and **14**. The two analogues **1** and **2** have been tested towards γ-glutamyltranspeptidase (γ-GT) and human glutathione S-transferase (hGST P1-1). Both analogues **1** and **2** are completely resistant to enzymatic degradation by γ-GT. The S-transferase utilizes the analogue **2** as a good substrate while is unable to bind the analogue **1**.

Introduction

The highly relevant and multifunctional roles played by glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) in a variety of biological processes—including xenobiotic detoxification and protection against radiations—continue to stimulate both theoretical and experimental studies.[1–8] Furthermore, the structural simplicity and the low molecular weight, render the GSH molecule an appealing target for chemical modification; thus, a very large number of structural analogues have been designed and synthesized.[9,10] As a result of these investigations a better understanding of the interactions with enzyme catalytic sites has been obtained and more potent, selective and stable inhibitors have been developed. In this research field we reported recently design and synthesis of a new group of GSH analogues characterized by chemical modifications centered at the level of the γ-Glu-Cys junction. By following this strategy the first examples of GSH pseudopeptide analogues, containing urea, urethane or sulfonamido groups replacing the native γ-glutamyl isopeptide bond, have been described.[11–13] Some of these analogues have subsequently been exploited to obtain enzyme inhibitors which are highly active and completely resistant to enzymatic degradation by γ-glutamyl-transpeptidase (γ-GT).[14] As an extension of these studies we report here synthesis and activity of the GSH analogues **1** and **2** in which the entire γ-glutamylic moiety is replaced by the residue of *cis* and *trans* 4-carboxy-L-proline, respectively (Fig. 1).

Proline is the only proteinogenic aminoacid to possess a naturally restricted conformation, characterized by the presence of a pyrrolidine ring in which the N–C^{α} bond is inserted; thus, use of the chiral and quite rigid proline ring as template to support side-chain functionalities typical of other aminoacids (namely proline–aminoacid chimeras) is an actively pursued approach to obtain peptidomimetics endowed with specific local restrictions

*Corresponding author. Tel.: +39-06-445-4218; fax: +39-06-491-491; e-mail: gino.lucente@uniroma1.it

0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00041-5

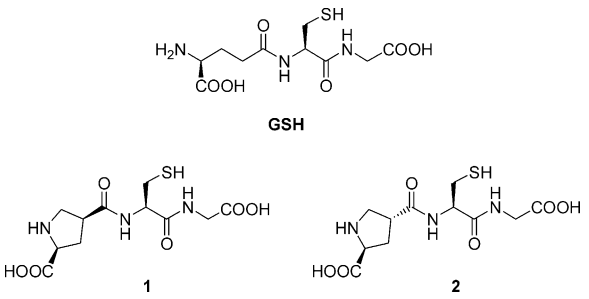


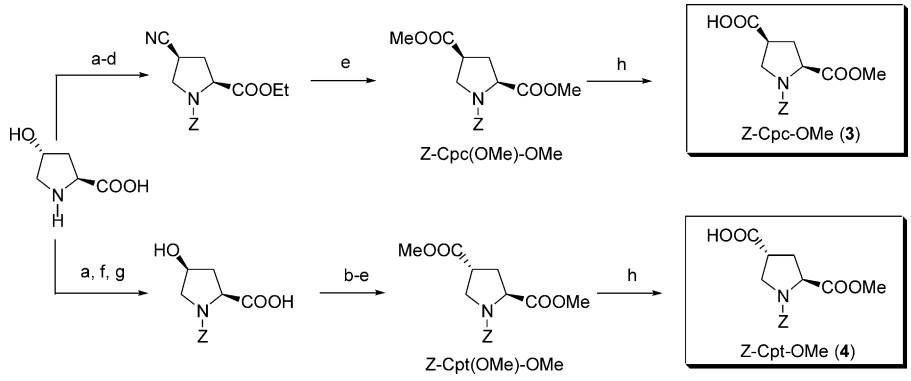
Figure 1. Structure of GSH and conformationally restricted GSH analogues **1** and **2**.

and improved metabolic stability.[15,16] Concerning proline-glutamate chimeras, a literature examination shows that, whereas *cis*- and *trans*-4-carboxy-L-prolines have been synthesized and examined for their activity in neurotransmission processes as glutamate conformer mimics,[17] their incorporation into biologically active peptides is very limited and no data are available concerning the use of proline as γ-glutamylic residue template.[18] Thus, compounds **1** and **2** represent the first reported examples of isopeptide analogues in which this modification strategy has been applied. Finally, it can be observed that both the new analogues maintain, as compared with the native GSH, all the functional groups capable to establish efficient non bonding interactions; the only difference, concerning these properties, is in fact represented by the presence of a secondary in place of a primary amino group at the N-terminal end of the molecule. Furthermore, the relevant conformational restriction imposed by the pyrrolidine ring on the backbone as well as the opposite spatial orientation of the amide γ-carbonyl in the two analogues may profoundly influence the reactivity towards the GSH dependent enzymes and render **1** and **2** useful probes to get information on the structural features of the involved active sites.

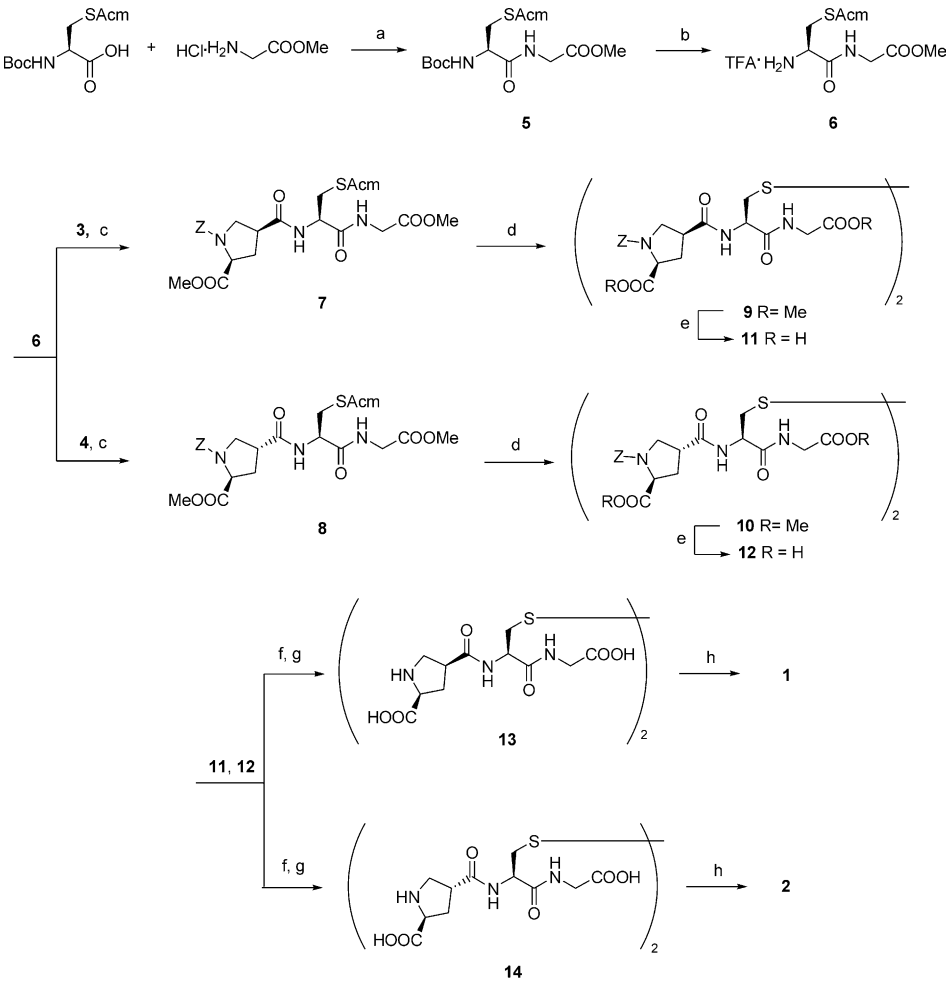
Results and Discussion

As useful route to the GSH analogues **1** and **2**, the incorporation of *cis*- and *trans*-4-carboxy-L-proline residues (Cpc and Cpt, respectively) into the Cys-Gly fragment was considered. Thus, the two diastereoisomeric *cis*- and *trans*-*N*-(benzyloxycarbonyl)-4-carboxy-L-proline-1-methyl esters *Z*-Cpc-OMe **3** and *Z*-Cpt-OMe **4** were synthesized as key intermediates (Scheme 1). According to the procedure previously reported by Bridges et al.[17] commercially available *trans*-4-hydroxy-L-proline was used as starting material to synthesize both *Z*-Cpc(OMe)-OMe and *Z*-Cpt(OMe)-OMe (Scheme 1). Subsequent regioselective hydrolysis of the C-4 ester group represents a critical step. After several attempts we found that a high selectivity and good yields of both the *cis* and *trans* isomer **3** and **4** can be obtained when the corresponding dimethyl esters are treated with equimolar amount of 1N NaOH in aqueous THF (Scheme 1). Structure as well as stereochemistry of monomethyl esters **3** and **4** are clearly reflected by their ^{1}H NMR spectra. Thus, when the spectrum of the *cis* monomethylester **3** is examined and compared with those of the related dimethyl esters, the singlet corresponding to the -OMe group at C-4 is absent and only the couple of singlets, which are typical of the -OMe group at the C-1 and are due, as expected, to restricted rotation around the proline CO–N bond, can be observed. Furthermore, the different resonance patterns exhibited by *cis* and *trans* monoesters allow exclusion of cross-contamination due to epimerisation at one of the two centers during the hydrolysis. Analogous considerations, deduced by the analysis of the ^{1}H NMR spectra, have been made concerning the stereochemistry of the *trans* monomethylester **4**.

Synthesis of the two conformationally restricted GSH analogues **1** and **2** were performed according to Scheme 2. In view of the mild protection conditions assured,[19] the acetamidomethyl (Acm) group was chosen to protect the cysteine thiol function. Accordingly, the Acm-derivatives **7** and **8** were synthesized by coupling *Z*-Cpc-OMe or *Z*-Cpt-OMe **3** and **4**, respectively, with Cys(Acm)-Gly-OMe trifluoroacetate **6** (Scheme 2) by following the solution phase peptide chemistry methodologies. ^{1}H NMR spectra of the two tripeptides **7** and **8** did not show evidence of diastereoisomeric species, thus confirming the stereochemical integrity of starting monoesters **3** and **4**. Selective removal (I_2 in MeOH, room temperature) of the Acm protecting group allowed direct conversion of **7** and **8** into the *N*-protected symmetrical disulfides **9** and **10**. Subsequent



Scheme 1. Reagents and conditions (see also ref 17): (a) $ClCO_2CH_2Ph$, $NaHCO_3$, H_2O, $PhCH_3$, 16 h, rt; (b) EtOH, *p*-TsOH, 16 h, reflux; (c) TsCl, py, 72 h, rt; (d) NaCN, DMSO, 80 °C, 4 h; (e) HCl, MeOH, 84 h, rt; (f) Jones reagent, acetone, 10 min, −5 °C then 2.5 h, rt; (g) $NaBH_4$, MeOH, 20 h, −5 °C; (h) THF, NaOH 1 N, 0.5 equiv, 30 min, 0 °C then 1 h, rt.



Scheme 2. Reagents and conditions: (a) DCCI, NMM, THF, 0 °C, 3 h then 5 °C, 16 h; (b) TFA, 1 h, rt; (c) isobutyl chloroformate, TEA, THF, −15 °C, 15 min then 0 °C, 2 h and 5 °C, 16 h; (d) I_2, MeOH, 4 h, rt; (e) NaOH 1 N, THF, 0 °C, 20 min then 16 h, rt; (f) HBr/CH_3COOH, 5 h, rt; (g) 2 N aq ammonia, rt, 20 min; (h) $(n\text{-But})_3P$, n-PrOH–H_2O, 3 h, rt.

removal in two steps of all protecting groups, followed by treatment of the resulting hydrobromides with aqueous ammonia gave the fully deprotected GSSG analogues **13** and **14** in good yields. The desired GSH thiol forms **1** and **2** were obtained by reductive cleavage of the disulfide link with tri-*n*-butylphosphine (1.2 equiv) in water/*n*-propanol solution, by operating at pH 8.5 (aqueous ammonia).[11] Mass spectra of **1** and **2** show the most abundant peaks corresponding to $M^{++}1$ species (320 m/z). The structures assigned to the GSH analogues **1** and **2** are in accordance with their spectroscopic properties (see Experimental). A characteristic feature of the ^{13}C NMR spectrum of **1** and **2** is represented by the resonance of the Cys β-carbon atom which is shifted at higher field (32.45 and 25.43 ppm) as compared with the corresponding atoms in the disulfide precursors **13** and **14** (38.90 and 38.87 ppm, respectively). This spectral behaviour represents, as recently noted,[20] a reliable diagnostic feature in order to distinguish between cysteine and cystine containing forms.

Activity on γ-Glutamyltranspeptidase (γ-GT)

γ-GT is a membrane bound enzyme which catalyzes the transfer of the γ-glutamyl residue of GSH and related γ-glutamyl compounds to a variety of acceptors such as aminoacids and dipeptides (transpeptidation) and water (hydrolysis).[21,22] Its proposed physiological role includes aminoacid and peptide transport as well as detoxification reactions through mercapturic acids formation. The reaction mechanism involves a covalent γ-glutamyl-enzyme intermediate and specificity studies have shown that the γ-glutamyl site (donor site) of the enzyme accepts many γ-glutamyl derivatives, including D-forms as well as α-methyl derivatives.[23] Other modifications of the γ-glutamyl backbone are found in glutamine-based affinity labels for the same enzyme site. The reported GSH analogues **1** and **2** show unusual structural and stereochemical alterations at the level of the γ-glutamyl moiety and could therefore interfere with the donor site of γ-GT or even act as substrates. Thus both **1** and **2** have been tested as putative inhibitors or substrates of the enzyme.

The enzymatic activity in the presence of 1 mM concentration of the substrate L-γ-glutamyl-*p*-nitroanilide (GPNA) is completely unaffected by the presence of compound **1** or **2** at 1 mM or 2 mM concentration even after preincubation at 37 °C of the enzyme with the putative inhibitor for 30 or 60 min.

Thus, at these concentrations the tested compounds are not inhibitors of the enzyme. Furthermore they do not show detectable affinity for the γ-glutamyl site, as would be if they were affinity labels or even substrates, since an apparent inhibition would be observed.

Activity on human glutathione *S*-transferase P1-1 (hGST P1-1)

The glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a superfamily of multifunctional enzymes involved in the cellular detoxification of xenobiotic compounds via a conjugation reaction with GSH which is highly specific for the GST active site.[24]

Enzymatic experiments were performed to check the ability of the hGST P1-1 to replace GSH with the GSH analogues **1** and **2**. In the hGST P1-1 catalysed nucleophilic reaction with the electrophilic substrate 1-chloro-2,4-dinitrobenzene (CDNB), the natural GSH was replaced either by GSH analogue **1** or **2**. In the presence of **1**, a specific activity not higher than 1.0 U/mg was observed up to 3 mM analogue concentration. This activity corresponds to about 1% of that found with the natural substrate GSH. The absence of any competitive inhibition toward GSH up to 10 mM concentration of analogue **1** (data not shown) suggests that the lack of activity is merely due to a scarce affinity for the GSH binding site and not to a non-productive binding of analogue **1**. On the contrary, compound **2** showed a quite good interaction with the hGST P1-1 active site and it is actively conjugated to CDNB. The dependence of the GST activity on the concentration of the GSH-analogue (Fig. 2), identifies a $K_m = 0.7 \pm 0.2$ mM, which is about 3.5-fold higher than the K_m for GSH ($K_m^{GSH} = 0.2$ mM). At saturating analogue concentration, the specific activity was 38 ± 3.4 U/mg, which is only 2-fold lower than the specific activity found with the nucleophilic substrate GSH (80 U/mg at pH 6.5 and 25 °C).

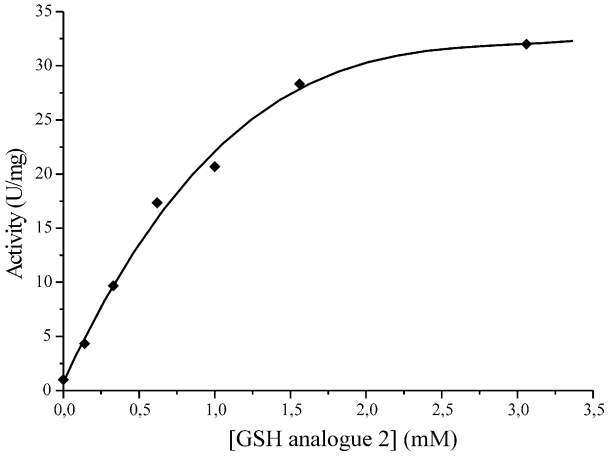


Figure 2. Conjugation reaction between CDNB and the GSH analogue **2**. The solid line is the best fit of the experimental data to a hyperbolic binding equation which fulfils a K_m value of 0.7 ± 0.2 mM and a V_{max} of 38 ± 3.4 U/mg.

Conclusions

In conclusion, we report here an efficient synthesis of the two novel diastereoisomeric glutathione analogues **1** and **2** containing the conformational rigid skeleton of *cis*- or *trans*-4-carboxy-L-proline residue, respectively, in place of the native γ-glutamyl moiety. At the best of our knowledge these analogues represent the first models in which the conformational freedom of native GSH has been reduced by introducing a ring equivalent of the glutamate residue.

As reported above the biological activity of analogues **1** and **2** has been tested on γ-GT and GST. Compounds **1** and **2** are not substrates or inhibitors on γ-GT; these results clearly show that the conformational restricted backbone of these analogues is apparently not compatible with binding to the donor site of γ-GT thus rendering these models resistant to the enzymatic degradation by this enzyme.

It is well known that the γ-glutamyl moiety is the main binding determinant for the GSH-GST interaction, giving multiple polar interaction with specific protein residues, that is Asp98, Gln64 and Ser65.[25] The here reported results indicate that the transoid spatial arrangement involving the C^{α} and C^{γ} pyrrolidine carboxy groups, present in the analogue **2,** represents the favoured conformation for binding to the GST active site. However, further investigations are requested in order to define which of the structural and conformational features distinguishing analogues **1** and **2** from the native GSH are specifically responsible of the observed binding selectivity at the enzymatic active site.

It should be remembered here that the GTSs superfamily may play an important part in cellular resistance to foreign, electrophilic toxins as well as to many anticancer drugs which are also electrophilic in nature.[26] In particular, the hGST P1-1 isoform represents an attractive target since it has been found to be over-expressed in a number of solid tumors, thus diminishing the effectiveness of cytotoxic drugs.[27] As a consequence, the versatily of the here described synthetic approach which relies upon the utilization of a chiral 4-carboxy-proline skeleton as glutamate conformer mimic could give rise to new interesting peptidase stable GSH inhibitors providing tumor-directed potentiation of conventional antineoplastic agents.

Experimental

Chemistry

Boc-Cys(Acm)-OH and H-Gly-OBu^t·HCl were obtained from Novabiochem. TLC was performed on Merck 60 F_{254} silica gel plates developed with the following solvent system: (a) $CHCl_3$/MeOH (95:5); (b) $CHCl_3$/*i*-PrOH (95:5); (c) *n*-BuOH/AcOH/H_2O (3:2:2). Column chromathography was carried out using Merck 60 silica gel (230–400 mesh). Optical rotations were taken at 25 °C with Perkin–Elmer 241 polarimeter. IR

spectra were recorded employing a Perkin–Elmer 1600 FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra were determined on a Varian VXR 300 MHz instrument (δ expressed in ppm). ^{13}C NMR data for compounds **1**, **2**, **13** and **14** were confirmed by APT experiments. The mass spectra of **1** and **2** have been obtained in electrospray (ES) conditions by a LCQ (Finnigan, S. Josè, CA, USA) instrument. The 60 μM solution of the sample in CH_3OH/H_2O 50:50 containing 1% AcOH has been directly injected at the flow rate of 5 μL/min. Abbreviations: THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; NMM, *N*-methylmorpholine; EtOAc, ethyl acetate; TEA, triethylamine; TFA, trifluoroacetic acid; DMSO, dimethylsulphoxide.

Z-Cpc-OMe (3) and Z-Cpt-OMe (4). *N*-(Benzyloxycarbonyl)-4-carboxy-L-proline dimethyl ester (3.2 g, 10 mmol) was dissolved in THF (21 mL) and 1 N NaOH (10 mL) was added dropwise at 0 °C with stirring. After 30 min at 0 °C and 1 h at room temperature, the reaction mixture was concentrated and treated with ether and saturated acqueous $NaHCO_3$. Acidification of aqueous layer with concentrated HCl caused precipitation of the oily product which was extracted into EtOAc. The residue obtained after drying and evaporation of the organic layer was chromatographed on silica gel using CH_2Cl_2/MeOH (99:1) as eluant to give the corrisponding monomethyl esters.

(3). Oil (2.0 g, 65%). R_f (a) = 0.39; $[\alpha]_D = -42°$ (*c* 1, $CHCl_3$); IR (nujol): 3500–2800 br, 1740, 1705, 1415 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 2.42 (1H, m, Cpc β-CH_A), 2.51 (1H, m, Cpc β-CH_B), 3.14 (1H, m, Cpc γ-CH), 3.55 and 3.72 (3H, s, OCH_3), 3.79–3.97 (2H, m, Cpc δ-CH_2), 4.41 (1H, m, Cpc α-CH), 5.02–5.21 (2H, m, $PhCH_2$), 6.50 (1H, br, COOH), 7.26–7.37 (5H, aromatics).

(4). Oil (2.0 g, 65%). R_f (a) = 0.46; $[\alpha]_D = -48.9°$ (*c* 1, $CHCl_3$); IR (nujol): 3500–2800 br, 1740, 1710, 1410 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 2.23 (1H, m, Cpt β-CH_A), 2.52 (1H, m, Cpt β-CH_B), 3.26 (1H, m, Cpt γ-CH), 3.59 and 3.76 (3H, s, OCH_3), 3.72 (1H, m, Cpt δ-CH_A), 3.89 (1H, m, Cpt δ-CH_B), 4.51 (1H, m, Cpt α-CH), 5.01–5.21 (2H, m, $PhCH_2$), 5.70 (1H, br, COOH), 7.26–7.36 (5H, aromatics).

Boc-Cys(Acm)-Gly-OMe (5). To a stirred solution of Boc-Cys(Acm)-OH (6.0 g, 20.5 mmol) in dry THF (8 mL) H-Gly-OMe·HCl (2.6 g, 20.5 mmol) and NMM (2.2 mL, 20.5 mmol) in dry THF (8 mL) were added at 0 °C followed by portionwise addition of DCC (4.2 g, 20.5 mmol) in dry THF (8 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 EtOAc and the organic layer washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$ and H_2O. The residue obtained after drying and evaporation was cromatographed on silica gel using $CHCl_3$/MeOH (95:5) as eluant to give dipeptide methylester **5** as a white foam (7.0 g, 94%). R_f (a) = 0.33; $[\alpha]_D = +26.5°$ (*c* 1, $CHCl_3$); IR (KBr): 3450, 3300, 1745, 1690, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 1.47 (9H, s, 3×CH_3), 2.00 (3H, s, CH_3CO), 2.90 (2H, m, Cys β-CH_2), 3.75 (3H, s, OCH_3), 3.90–4.15 (2H, m, Acm CH_2), 4.30 (1H, m, Cys α-CH), 4.45–4.55 (2H, m, Gly CH_2), 5.65 (1H, d, *J* = 7.5 Hz, Cys NH), 7.20 (1H, br t, Gly NH), 7.35 (1H, br t, Acm NH).

***Z*-Cpc[-Cys(Acm)-Gly-OMe]-OMe (7) and *Z*-Cpt[-Cys(Acm)-Gly-OMe]-OMe (8).** The above reported dipeptide ester **5** (3.3 g, 9.1 mmol) was dissolved in TFA (10 mL). After 1 h at room temperature the solution was evaporated to dryness and the residue repeatedly evaporated with ether to give TFA·H-Cys(Acm)-GlyOMe **6** (3.4 g, 100%) which was used without further purification.

To an ice-cold solution of *Z*-Cpc-OMe **3** or Z-Cpt-OMe **4** (2.3 g, 7.5 mmol) in dry THF (28 mL) TEA (1.1 mL, 7.5 mmol) and isobutyl chloroformate (1 mL, 7.5 mmol) were added under stirring. After 15 min at −15 °C, methyl ester **6** (7.5 mmol) in TEA (1.1 mL, 7.5 mmol) and dry THF (20 mL) were added to the mixture at −15 °C with stirring. After 2 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. Work up, as described for compound **5**, gave an oily product which was purified on silica gel using $CHCl_3$/MeOH (9:1) as eluant to yield the corresponding pure tripeptide metilesters.

(7). White foam (2.3 g, 55%). R_f (a) = 0.29; $[\alpha]_D = -33°$ (*c* 1, $CHCl_3$); IR (KBr): 3300 br, 3065, 1745, 1655, 1540 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 2.05 (3H, s, CH_3CO), 2.42 (1H, m, Cpc β-CH_A), 2.53 (1H, m, Cpc β-CH_B), 2.75–3.01 (2H, m, Cys β-CH_2), 3.05 (1H, m, Cpc γ-CH), 3.60 (1H, m, Cpc δ-CH_A), 3.73 (6H, s, 2×OCH_3), 3.97 (1H, m, Cpc δ-CH_B), 3.90–4.15 (2H, m, Acm CH_2), 4.40–4.60 (2H, m, Gly CH_2), 4.50 (1H, m, Cpc α-CH), 4.80 (1H, m, Cys α-CH), 5.02–5.21 (2H, m, $PhCH_2$), 7.15 (2H, br, Cys NH and Acm NH), 7.26–7.37 (5H, aromatics), 7.42 (1H, br t, Gly NH).

(8). White foam (2.8 g, 68%). R_f (a) = 0.32; $[\alpha]_D = -23.6°$ (*c* 1, $CHCl_3$); IR (KBr): 3310 br, 1745, 1655, 1540 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ = 2.05 (3H, s, CH_3CO), 2.22 (1H, m, Cpt β-CH_A), 2.52 (1H, m, Cpt β-CH_B), 2.70 (1H, m, Cys β-CH_A), 2.95 (1H, m, Cys β-CH_B), 3.15 (1H, m, Cpt γ-CH), 3.60 (3H, s, OCH_3), 3.68 (1H, m, Cpt δ-CH_A), 3.73 (3H, s, OCH_3), 3.91 (1H, m, Cpt δ-CH_B), 3.95–4.15 (2H, m, Acm CH_2), 4.30 (1H, m, Gly CH_A), 4.56 (1H, m, Cpt α-CH), 4.60 (1H, m, Gly CH_B), 4.80 (1H, m, Cys α-CH), 5.01–5.21 (2H, m, $PhCH_2$), 6.80 (1H, br t, *J* = 6.15 Hz, Gly NH), 6.97 (1H, br t, *J* = 7.90 Hz, Cys NH), 7.25–7.35 (5H, aromatics), 7.36 (1H, obs, Acm NH).

[*Z*-Cpc(-Cys-Gly-OMe)-OMe]$_2$ (9) and [*Z*-Cpt(-Cys-Gly-OMe)-OMe]$_2$ (10). To a stirred solution of the above reported *S*-protected tripeptide esters (2.2 g, 4 mmol) in MeOH (48 mL) I_2 (2.03 g, 8 mmol) in MeOH (24 mL) was added portionwise at room temperature during 45 min. After 4 h under stirring the reaction mixture was cooled at 0 °C and decoulorized with 1 N $Na_2S_2O_3$. The residue obtained after removal of the solvent was partioned between H_2O and EtOAc and the organic layer was washed with 1 N $Na_2S_2O_3$ and H_2O. Drying and evaporation followed by purification by

silica gel column chromatography ($CHCl_3$/*i*-PrOH 95:5 as eluant) of the resulting crude products afforded the corresponding pure disulfide esters.

(9). Foam (1.3 g, 34%). R_f (b) = 0.29; $[\alpha]_D = -18.5°$ (*c* 1, $CHCl_3$); IR (KBr): 3480, 3305, 1745, 1690, 1540 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 2.30 (2H, m, Cpc β-CH_A), 2.59 (2H, m, Cpc β-CH_B), 2.82 (2H, m, Cys β-CH_A), 3.01 (2H, m, Cys β-CH_B), 3.07 (2H, m, Cpc γ-CH), 3.55 (6H, s, OCH_3), 3.72 (2H, m, Cpc δ-CH_A), 3.76 (6H, s, OCH_3), 3.87 (2H, m, Cpc δ-CH_B), 4.00–4.15 (4H, m, Gly CH_2), 4.40 (2H, m, Cpc α-CH), 5.01–5.22 (4H, m, $PhCH_2$), 5.48 (2H, m, Cys α-CH), 7.10 (2H, br t, *J* = 10.45 Hz, Cys NH), 7.26–7.35 (10H, aromatics), 8.41 (2H, t, *J* = 4.95 Hz, Gly NH).

(10). Foam (1.6 g, 36%). R_f (b) = 0.29; $[\alpha]_D = -26.1°$ (*c* 1, $CHCl_3$); IR (KBr): 3465, 3305, 1750, 1690, 1540 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 2.16 (2H, m, Cpt β-CH_A), 2.58 (2H, m, Cpt β-CH_B), 2.78–2.97 (4H, m, Cys β-CH_2), 3.15 (2H, m, Cpt γ-CH), 3.67 (6H, s, OCH_3), 3.74 (6H, s, OCH_3), 4.00 (4H, m, Gly CH_2), 4.02 (4H, m, Cpt δ-CH_2), 4.45 (2H, m, Cpt α-CH), 5.01–5.18 (4H, m, $PhCH_2$), 5.52 (2H, m, Cys α-CH), 6.94 (2H, t, *J* = 6.60 Hz, Cys NH), 7.25–7.35 (10H, aromatics), 8.37 (2H, m, Gly NH).

[*Z*-Cpc(-Cys-Gly-OH)-OH]$_2$ (11) and [*Z*-Cpt(-Cys-Gly-OH)-OH]$_2$ (12). The protected disulfide (1.2 g, 1.25 mmol) was dissolved in dry THF (3.1 mL) and 1 N NaOH (5.6 mL) was added under stirring at 0 °C. After 30 min at 0 °C and 18 h at room temperature, the pH was adjusted to 6.0 by 1 N $KHSO_4$ and the solution evaporated in vacuo to dryness and dried over KOH. The resulting residue gave the corresponding TLC-pure disulfide acids which were used as such.

(11). Foam (1.1 g, 96%). R_f (c) = 0.57; $[\alpha]_D = -17.5°$ (*c* 1, H_2O); IR (KBr): 3600–3100, 1700, 1675, 1540 cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.15 (2H, m, Cpc β-CH_A), 2.42 (2H, m, Cpc β-CH_B), 2.90 (2H, m, Cys β-CH_A), 2.95–3.15 (4H, m, Cys β-CH_B and Cpc γ-CH), 3.35 (4H, m, Cpc δ-CH_2), 3.75 (4H, m, Gly CH_2), 4.20 (2H, m, Cpc α-CH), 4.55 (2H, m, Cys α-CH), 5.01–5.23 (4H, m, $PhCH_2$), 7.26–7.35 (10H, aromatics), 8.35 (4H, br, Gly NH and Cys NH).

(12). Foam (1.4 g, 98%). R_f (c) = 0.69; $[\alpha]_D = -39.8°$ (*c* 1, H_2O); IR (KBr): 3600–3100, 1700, 1675, 1540 cm^{-1}; ^1H NMR (D_2O) δ = 1.99 (2H, m, Cpt β-CH_A), 2.30 (2H, m, Cpt β-CH_B), 2.70 (2H, m, Cys β-CH_A), 3.05–3.25 (4H, m, Cys β-CH_B and Cpt γ-CH), 3.45–3.75 (8H, m, Cpt δ-CH_2 and Gly CH_2), 4.15 (2H, m, Cpt α-CH), 4.60 (2H, m, Cys α-CH), 4.90–5.10 (4H, m, $PhCH_2$), 7.25–7.35 (10H, aromatics), 8.35 (4H, br, Gly NH and Cys NH).

[H-Cpc(-Cys-Gly-OH)-OH]$_2$ (13) and [H-Cpt(-Cys-Gly-OH)-OH]$_2$ (14). A solution of the above reported *N*-protected disulfide acid (1.0 g, 1.1 mmol) was treated with 30% HBr in glacial AcOH (20 mL) at room temperature. After 5 h the solution was evaporated under reduced pressure below 40 °C and the residue was dried over KOH. The obtained oil was dissolved in anhydrous MeOH; precipitation with anhydrous ether yielded [HBr Pro(-Cys-Gly-OH)-OH]$_2$ in quantitative yield which was used as such.

The hydrobromide (0.8 g, 1.0 mmol) was dissolved in 2 N aqueous NH_3 (18 mL) at room temperature. After 2 h, the aqueous solution was concentrated and subjected to column chromatography on Sephadex LH-20 using H_2O/MeOH (2:1) as eluant to afford the corresponding disulfides.

(13). Foam (0.6 g, 85%). R_f (c) = 0.54; $[\alpha]_D = -30.7°$ (*c* 1, H_2O); IR (KBr): 3240 br, 1680–1530, 1395, 1250 cm^{-1}; ^1H NMR (D_2O) δ = 2.10 (2H, m, Cpc β-CH_A), 2.62 (2H, m, Cpc β-CH_B), 2.75–2.98 (4H, m, Cys β-CH_2), 3.26 (2H, m, Cpc γ-CH), 3.37–3.56 (4H, m, Cpc δ-CH_2), 3.65 (4H, AB q, *J* = 18.0 Hz, Gly CH_2), 4.08 (2H, m, Cpc α-CH), 4.46 (2H, m, Cys α-CH); ^{13}C NMR (D_2O) δ = 33.07 (Cpc C^β), 38.90 (Cys C^β), 42.84 (Cpc C^γ), 43.51 (Gly C^α), 47.56 (Cpc C^δ), 53.02 (Cys C^α), 61.39 (Cpc C^α), 171.79, 173.32, 173.66 and 176.38 (4×CO).

(14). Foam (0.7 g, 70%). R_f (c) = 0.32; $[\alpha]_D = -32.4°$ (*c* 1, H_2O); IR (KBr): 3300 br, 1690–1540, 1390, 1250 cm^{-1}; ^1H NMR (D_2O) δ = 2.35 (4H, m, Cpt β-CH_2), 2.83 (2H, m, Cys β-CH_A), 3.11 (2H, m, Cys β-CH_B), 3.24 (2H, m, Cpt γ-CH), 3.39 (2H, m, Cpt δ-CH_A), 3.51 (2H, m, Cpt δ-CH_B), 3.62 (4H, s, Gly CH_2), 4.14 (2H, m, Cpt α-CH), 4.61 (2H, m, Cys α-CH); ^{13}C vNMR (D_2O) δ = 33.09 (Cpt C^β), 38.87 (Cys C^β), 42.47 (Cpt C^γ), 43.62 (Gly C^α), 47.93 (Cpt C^δ), 52.90 (Cys C^α), 61.44 (Cpt C^α), 171.61, 173.89, 173.97 and 176.33 (4×CO).

H-Cpc(-Cys-Gly-OH)-OH (1) and H-Cpt(-Cys-Gly-OH)-OH (2). A solution of the foregoing disulfide acid (0.4 g, 0.6 mmol) in a mixture of *n*-PrOH/H_2O (2:1) (12 mL) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. Tri-*n*-butyl phosphine (0.15 g, 0.72 mmol) added and the stoppered flask stirred at room temperature. After 3 h the reaction mixture was repeatedly washed with $CHCl_3$ and the pH of the aqueous solution adjusted to 6.0 by 1 N HCl. The solution was concentrated and subjected to column chromatography on Sephadex using H_2O/MeOH (2:1) as eluant to afford the corresponding reduced compounds.

(1). Vitrous foam (0.3 g, 98%). R_f (c) = 0.39; $[\alpha]_D = -27.9°$ (*c* 1, H_2O); IR (KBr): 3250 br, 1690–1530, 1395, 1255 cm^{-1}; ^1H NMR (D_2O) δ = 2.11 (1H, m, Cpc β-CH_A), 2.59 (1H, m, Cpc β-CH_B), 2.74 (1H, m, Cys β-CH_A), 2.88 (1H, m, Cys β-CH_B), 3.24 (1H, m, Cpc γ-CH), 3.36–3.54 (2H, m, Cpc δ-CH_2), 3.83 (2H, Gly CH_2), 4.15 (1H, m, Cpc α-CH), 4.43 (1H, m, Cys α-CH); ^{13}C NMR (D_2O) δ = 32.45 (Cys C^β), 32.92 (Cpc C^β), 41.52 (Gly C^α), 42.71 (Cpc C^γ), 47.70 (Cpc C^δ), 52.98 (Cys C^α), 60.89 (Cpc C^α), 172.56, 172.95, 173.46 and 173.70 (4×CO); MS (*m*/*z*) 320 ($M^+ + 1$).

(2). Vitrous foam (0.3 g, 90%). R_f (c) = 0.43; $[\alpha]_D = -28.9°$ (*c* 1, H_2O); IR (KBr): 3300 br, 1695–1540,

1340, 1250 cm^{-1}; ^1H NMR (D_2O) $\delta = 2.26$ (2H, m, Cpt β-CH_2), 2.73 (2H, m, Cys β-CH_2), 3.16 (1H, m, Cpt γ-CH), 3.30–3.47 (2H, m, Cpt δ-CH_2), 3.57 (2H, Gly CH_2), 4.10 (1H, m, Cpt α-CH), 4.34 (1H, m, Cys α-CH); ^{13}C NMR (D_2O) $\delta = 25.43$ (Cys C^{β}), 32.97 (Cpt C^{β}), 42.18 (Cpt C^{γ}), 43.28 (Gly C^{α}), 47.68 (Cpt C^{δ}), 55.65 (Cys C^{α}), 61.07 (Cpt C^{α}), 171.53, 173.85, 173.95 and 176.37 (4×CO); MS (*m*/*z*) 320 ($M^+ + 1$).

Biological assays

Bovine kidney γ-GT (about 26 U/mg), GPNA, H-Gly-Gly-OH and CDNB were purchased from Sigma. In vitro assay of γ-GT activity was performed according to Meister et al.[28] at saturating concentration of the acceptor molecule (H-Gly-Gly-OH = 150 mM).

Human placenta GST P1-1 (EC 2.5.1.18) was expressed in *Escherichia coli* and purified as previously described.[29] Enzymatic experiments concerning the activity of GSH analogues **1** and **2** on hGST P1-1 were performed at 25 °C in the presence of 1 mM CDNB as acceptor substrate. GST P1-1 activity was measured in 1 mL of 0.1 M K-phosphate buffer pH 6.5 containing 1 mM CDNB, 0.1 mM EDTA and variable amounts of either GSH or GSH analogue (**1** or **2**) ranging from 0.1 to 3 mM. Activity was recorded at 340 nm, where the DNB-conjugate absorbs ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$), using a double-beam Uvikon spectrometer thermostatted at 25 °C. One unit of GST activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of DNB-conjugate per min at 25 °C.

Acknowledgements

This work was partially supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MIUR, 40%, Italy).

References and Notes

1. Lyon, R. P.; Atkins, W. M. *J. Am. Chem. Soc.,* **2001**, *123*, 4408.
2. Rauk, A.; Armstrong, D. A.; Berges, J. *Can. J. Chem.* **2001**, *79*, 405.
3. Diaz, S.; Gonzáles, A.; Gonzáles de Riancho, S.; Rodríguez, A. *J. Organomet. Chem.* **2000**, *610*, 25.
4. Duckert, J. F.; Balas, L.; Rossi, J. C. *Tetrahedron Lett.* **2001**, *42*, 3709.
5. Cavero, M.; Hobbs, A.; Madge, D.; Motherwell, W. B.; Selwood, D.; Potier, P. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 641.
6. Burg, D.; Filippov, D. V.; Hermanns, R.; van der Marel, G. A.; van Boom, J. H.; Mulder, G. J. *Bioorg. Med. Chem.* **2002**, *10*, 195.
7. Burg, D.; Hameetman, L.; Filippov, D. V.; van der Marel, G. A.; Mulder, G. J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1579.
8. Li, Y.; Carter, D. E.; Mash, E. A. *Synthetic Commun.* **2002**, *32*, 1579.
9. Douglas, K. T. In *Offprints from Glutathione: Chemical, Biochemical and Medical Aspects*; Dolphin, D.; Poulson, R.; Avramovic, O. Eds.; Wiley & Sons: New York, 1989; Part A.
10. Lucente, G.; Luisi, G.; Pinnen, F. *Il Farmaco* **1998**, *53*, 721.
11. Calcagni, A.; Duprè, S.; Lucente, G.; Luisi, G.; Pinnen, F.; Rossi, D. *Int. J. Pept. Protein Res.* **1995**, *46*, 434.
12. Calcagni, A.; Duprè, S.; Lucente, G.; Luisi, G.; Pinnen, F.; Rossi, D.; Spirito, A. *Arch. Pharm. Pharm. Med. Chem.* **1996**, *329*, 498.
13. Luisi, G.; Calcagni, A.; Pinnen, F. *Tetrahedron Lett.* **1993**, *34*, 2391.
14. Vince, R.; Brownell, J.; Akella, L. B. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 853.
15. Sharma, R.; Lubell, W. D. *J. Org. Chem.* **1996**, *61*, 202, and references cited therein.
16. Kitas, E. A.; Löeffer, B. M.; Daetwyler, S.; Dehmlow, H.; Aebi, J. D. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1727.
17. Bridges, R. J.; Stanley, M. S.; Anderson, M. W.; Cotman, C. W.; Chamberlin, A. R. *J. Med. Chem.* **1991**, *34*, 717.
18. Herman, J. P. Eur. Patent 0 334 244 A2, 1989. *Chem. Abstr.* **1990**, *112*, 119449.
19. Kamber, B. *Helv. Chim. Acta* **1971**, *54*, 927.
20. Sharma, D.; Rajarathnam, K. *J. Biomol. NMR* **2000**, *18*, 165.
21. Meister, A.; Tate, S. S. *Ann. Rev. Biochem.* **1976**, *45*, 559.
22. Tate, S. S.; Meister, A. *Mol. Cell. Biochem.* **1981**, *39*, 357.
23. Douglas, K. T. *Adv. Enzymol.* **1987**, *59*, 103.
24. Armstrong, R. N. *Chem. Res. Toxicol.* **1997**, *10*, 2.
25. Oakley, A. J.; Lo Bello, M.; Battistoni, A.; Ricci, G.; Rossjohn, J.; Villar, H. O.; Parker, M. W. *J. Mol. Chem.* **1997**, *274*, 84.
26. Salinas, A. M.; Wong, M. G. *Curr. Med. Chem.* **1999**, *6*, 279, and references cited therein.
27. O'Brien, M. L.; Tew, K. D. *Eur. J. Cancer* **1996**, *32A*, 967.
28. Meister, A.; Tate, S. S.; Griffith, O. W. In *Methods Enzymology*; Jacoby, W. B., Ed.; Academic: New York, 1981; Vol. 77, p 237.
29. Ricci, G.; Del Boccio, G.; Pennelli, A.; Lo Bello, M.; Petruzzelli, R.; Caccuri, A. M.; Barra, D.; Federici, G. *J. Biol. Chem.* **1991**, *266*, 21409.