Synthesis and Activity of the Glutathione Analogue γ-(L-γ-Oxaglutamyl)-L-cysteinyl-glycine

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Summary

An efficient synthesis of the backbone modified glutathione analogue γ-(L-γ-oxaglutamyl)-L-cysteinyl-glycine (**7**), characterized by the presence of an urethane O-CO-NH linkage replacing the γ-glutamylic CH_2CO-NH fragment is described. The new analogue has been fully characterized by ^1H- and ^{13}C-NMR, and FAB-MS. Compound **7** was tested for inhibition of γ-glutamyltransferase activity and was found to be a non-competitive inhibitor of hog kidney γ-glutamyltransferase (EC 2.3.2.2).

Introduction

In recent years there has been increased interest in the structural modifications of biologically active peptides in order to influence potency, selectivity, and metabolic stability [1,2]. In the context of our studies on this subject we focused our attention on backbone-modified glutathione (GSH) analogues. Although the high biological relevance and the structural simplicity render the GSH molecule an appealing target for chemists, an analysis of the literature reveals that the majority of the chemical modifications performed on this tripeptide does not involve the backbone atoms but is centered on the cysteine side chain and peripherical functional groups [3-6]. Notable exceptions are represented by the synthesis of an end-group modified retro-inverso isomer [7] and of a pseudopeptide analogue containing a sulfonamide junction [8].

More recently [9] we started a research program aimed at studying GSH analogues characterized by two fundamental properties: i) close resemblance with the natural substrate in terms of both molecular size and functional group arrangement and ii) altered structure and reactivity of the γ-glutamylic CO-NH junction obtained through chemical modification or replacement of the γ-CH_2 bound to the amide carbonyl group.

The first property should play a favorable role during the molecular recognition step, thus rendering these models suitable tools for the study of the reaction mechanism of different enzymatic systems involved in the glutamylic cycle. The chemical alteration of the glutamylic γ-CH_2 represents, on the other hand, a versatile strategy for introducing relevant consequences at both the conformational and biochemical level. The peculiar properties of the CO-NH group such as polarity, H bond capacity, double bond character, and metabolic stability can be in fact profoundly altered by changing the nature of the group directly bound to the amide carbonyl.

This approach has been recently adopted in the synthesis of the backbone-modified GSH analogue γ-(L-γ-azaglutamyl)-L-cysteinyl-glycine, H-Glz(-Cys-Gly-OH)-OH, characterized by the presence of a NH-CO-NH urea linkage deriving from the replacement of the native Glu γ-CH_2 with the NH group [9].

As a continuation of our studies we report here the synthesis and properties of the new GSH analogue **7**. This model is obtained by linking through a carbonyl group a serine residue to the Cys-Gly dipeptide. Thus, the GSH glutamylic CH_2CO-NH fragment is replaced by the urethane O-CO-NH junction. From a formal point of view the new GSH analogue contains the L-γ-oxaglutamic acid (Glo) residue and can be indicated as γ-(L-γ-oxaglutamyl)-L-cysteinyl-glycine and abbreviated as H-Glo(-Cys-Gly-OH)-OH **7** (Fig. 1).

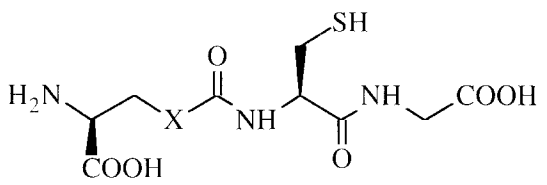

Figure 1

X = CH_2	H-Glu(-Cys-Gly-OH)-OH (GSH)
X = NH	H-Glz(-Cys-Gly-OH)-OH (see Ref.[9])
X = O	H-Glo(-Cys-Gly-OH)-OH **7**

In order to evaluate the putative biological activity of **7**, inhibition studies have been performed on the enzyme γ-glutamyltransferase (EC 2.3.2.2). This enzyme, which is the subject of continuing research in order to establish its reaction mechanism [10,11], catalyzes the transfer of the γ-glutamyl moiety from many donor molecules including GSH [12] to a large number of acceptor substrates [13,14] *via* a γ-glutamyl-enzyme intermediate in which a serine residue of the enzyme forms an ester linkage with the γ-glutamyl group [11]. By taking into account the structural similarity between **7** and the GSH parent, a correspondent linkage between the enzyme and the γ-oxaglutamyl group, consistent with a O-CO-O carbonate, could be involved for **7**.

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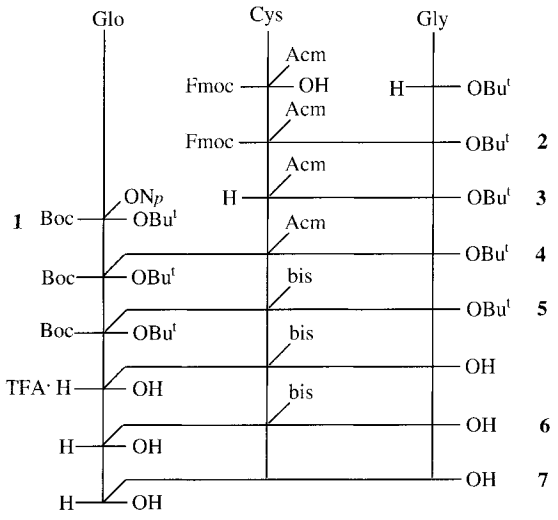
Results and Discussion

Chemical Synthesis

As useful strategy leading to **7**, the incorporation of a β-derivatized seryl residue onto the Cys-Gly fragment was considered. In view of the mild deprotection conditions assured [15], the acetamidomethyl (Acm) group was chosen to protect the cysteine thiol function. The ethyl ester protection of the carboxy functions was discarded owing to difficulties encountered during the hydrolysis of the *N*-protected glutathione disulfide tetraethyl ester. To achieve satisfactory yields, the use of the *tert*-butyl esters, removable under acidic conditions, was necessary. Thus (see Scheme 1), the active carbonate Boc-Glo(ON*p*)-OBu^t **1**, obtained by acylation of Boc-Ser-OBu^t with *p*-nitrophenyl chloroformate, was coupled with H-Cys(Acm)-Gly-OBu^t **3** at 80° for 10 h, to give Boc-Glo[-Cys(Acm)-Gly-OBu^t]-OBu^t **4** in 80% yield. Selective removal under mild conditions (I_2 in MeOH, room temperature) of the Acm protecting group allowed direct conversion of **4** into the symmetrical disulfide tetra*t*-butyl ester (68% yield). Subsequent removal in a single step of all protecting groups from **5** using trifluroacetic acid (TFA) at room temperature, followed by treatment of the resulting *bis*-trifluoroacetate with aqueous ammonia, gave the fully deprotected glutathione disulfide (GSSG) analogue **6** in high yield. Reductive cleavage of the disulfide link in **6** was effected with a small excess (1.2 equiv.) of tri-*n*-butylphosphine [9]. All the starting material disappeared after 1 h at room temperature and the yield of the desired GSH analogue **7** was practically quantitative.



Scheme 1. Synthesis of H-Glo(-Cys-Gly-OH)-OH **7**.

The structure assigned to the γ-oxa-analogues **6** and **7** is in accordance with the spectroscopic properties; in Table 1 a comparison of the ^{1}H- and ^{13}C-NMR data with those of GSSG and GSH parents is reported. In the ^{1}H-NMR spectra, in agreement with the different environment, the Glo β-CH_2 signals of **6** and **7** appear shifted downfield (4.35 and 4.45 δ, respectively) as compared with the corresponding Glu β-CH_2 resonances of the GSSG and GSH (2.05 and 2.1 δ, respectively). ^{13}C-NMR spectra (D_2O) of both **6** and **7** reveal four carbonyl signals, one of them (159.73 δ for **6**; 159.81 δ for **7**) consistent with the presence of the O-CO-NH carbonyl group. The GSH Cys C$^\beta$ signal shows a characteristic high field shift (13 ppm *ca.*) relative to the same carbon atom in the disulfide precursor GSSG [16]; the same strong effect is observed in the case of the new analogues **6** and **7**. The Gly CH_2 shift differences between the natural compounds and the new analogues **6** and **7** seem to indicate distinct backbone conformations caused by the amide *versus* urethane replacement.

Biochemical Studies

Inhibition studies concerning compound **7** have been performed on the enzyme γ-glutamyltransferase from hog kidney and compared to those of the parent GSH and the γ-aza-analogue [9]. The inhibition has been studied first on the γ-glutamyl binding site (donor site). According to previous work [9], the reaction was initially performed under pseudo-first order conditions, at saturating concentrations of the acceptor molecule ([H-Gly-Gly-OH] = 150 mM). A non-competitive inhibition pattern was observed (Figure 2A) at donor molecule (L-γ-glutamyl-*p*-nitroanilide, GPNA) concentrations between 0.1 and 1.0 mM. Linear double reciprocal plots of initial velocity data are obtained. From the linear plot of

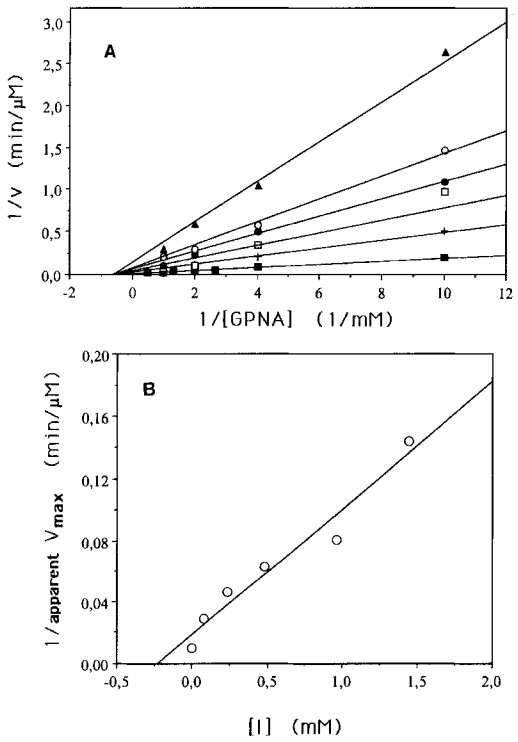


Figure 2. Inhibition of hog kidney γ-glutamyltransferase by compound **7** at pH 8.0, 30 °C, 0.1 M Tris-HCl buffer. Enzyme concentration: 35 mU/ml. (A) Lineweaver-Burk plot of the inhibition by binding at the donor site with GPNA as donor substrate (0.1–1 mM) and 150 mM H-Gly-Gly-OH as acceptor substrate. Concentration of compound **7** was 0 (■); 0.08 (✚); 0.24 (●); 0.48 (❑); 0.96 (○), and 1.44 mM (▲). (B) Plot of intercepts on the 1/*v* axis (1/apparent V_{max}) against inhibitor concentration. Data are obtained from (A). From the intercept with the *x* axis a value of $K_i = 2.5 \times 10^{-4}$ M is calculated.

Table 1. ^{1}H- and ^{13}C-NMR data* for GSSG, GSH, and their γ-oxa analogues **6** and **7**.

	[H-Glu(-Cys-Gly-OH)-OH]$_2$ (GSSG)		H-Glu(-Cys-Gly-OH)-OH (GSH)		[H-Glo(-Cys-Gly-OH)-OH]$_2$ **6**		H-Glo(-Cys-Gly-OH)-OH **7**	
Residue	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Glu or Glo								
C^{α}	3.7	56.59	3.75	56.55	3.9	56.73	4.0	56.78
C^{β}	2.05	28.85	2.1	28.82	4.35	66.67	4.45	66.68
C^{γ}(O)	2.45	34.06	2.5	34.00	-	-	-	-
CO-O	-	176.24	-	176.20	-	173.88	-	173.99
CO-N (O-CO-N)	-	177.52	-	177.56	-	159.73	-	159.81
Cys								
C^{α}	4.65	55.36	4.5	58.41	4.35	56.93	4.3	59.63
C^{β}	2.85 (H_A) 3.15 (H_B)	41.52	2.85	28.23	2.8 (H_A) 3.15 (H_B)	41.96	2.9	28.67
CO	-	175.22	-	175.13	-	175.13	-	174.86
Gly								
C^{α}	3.9	44.40	3.9	44.26	3.7	45.73	3.7	46.15
CO	-	177.51	-	176.25	-	178.31	-	179.07

* In D_2O at 23 °C. Chemical shifts are relative to internal dioxane.

intercepts on the $1/v$ axis (1/apparent V_{max}) against the inhibitor concentration a value of $K_i = 2.5 \times 10^{-4}$ M is calculated (Figure 2B).

The stability of the analogue **7** has been tested by monitoring through amino acid analysis the appearance of serine as the end product of either enzymatic or spontaneous hydrolysis. The activity of the enzyme on compound **7** is less than 0.05% of that on the natural substrate GSH. The non enzymatic hydrolysis at pH 8.0 and room temperature accounts for about 0.5% of the total amount of **7**, after two hours monitoring.

The inhibition experiments on the Cys-Gly site (acceptor site), performed at fixed donor substrate concentration of 1.0 mM, give data which do not fit in linear plots, denoting a more complex interaction with the enzyme (data not shown). On the contrary, both GSH and the previously studied γ-aza analogue showed a different inhibition pattern, giving competitive inhibition on the donor site with K_i values of similar order. It is worth noting that on the acceptor site an uncompetitive inhibition was observed for the γ-aza-analogue and a non-competitive inhibition with GSH as inhibitor [9].

Conclusion

In this paper an efficient synthesis leading to a GSH analogue containing an urethane junction in place of the γ-glutamylic amide bond has been described. The operated replacement should confer more rigidity to the backbone due to the participation of the oxygen atom to mesomeric effects with the directly bonded CO-NH. A literature examination confirms this and indicates well defined preferred conformations adopted by urethane backbone linkages [17, 18, 19]. Thus, the new analogue **7**, as compared with both the native GSH and the previously studied urea bond-containing derivative [9], is likely to adopt distinct solution conformations as well as different interactions with the appropriate area of the enzyme; the different inhibition patterns observed for the two GSH analogues modified at the γ-CH_2 atom seem to reflect these interaction differences. The possible contribution to the inhibition process of an acyl-enzyme intermediate chemically different from the normal γ-glutamyl-enzyme [*i.e.* Enzyme-CH_2-O-CO-O-CH_2-CH(NH_2)COOH instead of Enzyme-CH_2-O-CO-CH_2-CH_2-CH(NH_2)COOH] and its role into the inhibition mechanism is at the present under study in our

laboratory. The versatility of the here described synthetic approach which relies upon the utilization of chiral β-substituted α-amino acids can give rise to a family of new substrates suitable to define the conformational and chemical aspects involved in the inhibition and interaction mechanism.

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Experimental Part

Chemistry

TLC was performed on Merck 60 F_{254} silica gel plates. 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. Elemental analyses (C, H, N and S) were performed on a Carlo Erba model 1106 analyzer and were within ±0.4% of the theoretical values. ^{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 **6** and **7**, and relative to internal tetramethylsilane for the other compounds. The assignment of resonances in Table 1 were confirmed by 2D ^{1}H-^{13}C hetero-correlated long-range experiments [20] present in the instrument (Varian XL-300) routine. Fast atom bombardment (FAB) mass spectrum of compound **7** was recorded on a VG 70-70 EQ-MF instrument equipped with a standard FAB source (8 keV, Xe atoms, glycerol/1-thioglycerol solution).

The abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCCI, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxy-benzotriazole; NMM, *N*-methylmorpholine; N*p*, *p*-nitrophenyl; THF, tetrahydrofuran. Boc-Ser-OBu^t, HCl·H-Gly-OBu^t and Fmoc-Cys(Acm)-OH were obtained from Bachem.

Boc-Glo(ONp)-OBu^t (**1**)

To a stirred solution of Boc-Ser-OBu^t (6.8 g, 26.0 mmol) in pyridine (50 mL) *p*-nitrophenyl chloroformate (5.2 g, 26.0 mmol) was added at room temperature. After 24 h at 40 °C the reaction mixture was evaporated under reduced pressure and the residue was taken up in $CHCl_3$. The solution was repeatedly and successively washed with ice-cold 1 N $KHSO_4$, saturated aqueous Na_2CO_3 and H_2O. The residue obtained after drying and evaporation was chromatographed on silica gel using $CHCl_3$: MeOH (99:1) as eluant to give carbonate **1** as an oil (8.8 g, 79%).– $[\alpha]_D = +14.0°$ (c = 1, $CHCl_3$).– R_f 0.8 in $CHCl_3$/MeOH (99:1).– IR ($CHCl_3$): 3425, 1770, 1730-1710, 1500 cm^{-1}.– ^{1}H-NMR ($CDCl_3$) δ = 1.9 (9 H, s, 3 × CH_3), 2.0 (9H, s, 3 × CH_3), 4.55 (3H, m, Glo α-CH and β-CH_2), 5.35 (1H, d, J = 6.5 Hz, NH), 7.35 (2H, m, aromatic) and 8.25 (2H, two lines, aromatic).– Anal. ($C_{19}H_{26}N_2O_9$).

Fmoc-Cys(Acm)-Gly-OBu^t (**2**)

Fmoc-Cys(Acm)-OH (5.0 g, 12.0 mmol) was dissolved in THF (30 mL) and HOBt (1.6 g, 12.0 mmol) was added with stirring. The solution was cooled to 0 °C and an ice-cold solution containing HCl·H-Gly-OBu^t (2.0 g, 12.0 mmol) and NMM (1.2 g, 12.0 mmol) in THF (20 mL) was added, followed by portionwise addition of a solution of DCCI (2.5 g, 12.0 mmol) in THF (10 mL). 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. 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 chromatographed on silica gel using $CHCl_3$:MeOH (98:2) as eluant to give *t*-butyl ester **2** as a foam (5.9 g, 93%).– $[\alpha]_D = -25.0°$ (c = 1, DMF).– R_f 0.65 in $CHCl_3$/MeOH (97:3).– IR ($CHCl_3$): 3440, 3330, 1725, 1670-1650, 1510 cm^{-1}.– ^{1}H-NMR ($CDCl_3$) δ = 1.45 (9H, s, 3 × CH_3), 2.0 (3H, s, CH_3CO), 2.9 (2H, m, Cys β-CH_2), 3.95 (2H, m, Acm CH_2), 4.2-4.5 (3H, m, Fmoc CH-CH_2), 4.25-4.65 (3H, Cys α-CH and Gly CH_2), 6.15 (1H, d, J = 7.0 Hz, Cys NH), 7.15 (1H, br. t, Gly NH), 7.3 (1H, obscured, Acm NH), 7.25-7.8 (8H, m, aromatic).– Anal. ($C_{27}H_{33}N_3O_6S$).

Cys(Acm)-Gly-OBu^t (**3**)

To a solution of the above reported *t*-butyl ester **2** (5.6 g, 10.6 mmol) in CH_2Cl_2 (80 mL) DBU (1.6 g, 10.6 mmol) was added at room temperature. After 15 min the solution was evaporated to dryness and the residue chromatographed on silica gel using $CHCl_3$:MeOH (9:1) as eluant to give pure *N*-deprotected dipeptide ester **3** as an oil (2.9 g, 90%).– $[\alpha]_D = -18.0°$ (c =1, DMF).– R_f 0.5 in $CHCl_3$/MeOH (9:1).– IR ($CHCl_3$): 3360 br., 1730, 1670, 1660 cm^{-1}.– ^{1}H-NMR ($CDCl_3$) δ = 1.4 (9H, s, 3 × CH_3), 1.95 (3H, s, CH_3CO), 2.15 (2H, br., Cys NH_2), 2.9 (2H, m, Cys β-CH_2), 3.6 (1H, m, Cys α-CH), 3.9 (2H, m, Acm CH_2), 4.3 (2H, m, Gly CH_2), 7.25 (1H, br. t, Gly NH), 7.9 (1H, br. t, Acm NH).– Anal. ($C_{12}H_{23}N_3O_4S$).

Boc-Glo[-Cys(Acm)-Gly-OBu^t]-OBu^t (**4**)

The above reported dipeptide *t*-butyl ester **3** (2.8 g, 9.2 mmol) was dissolved in dioxane (20 mL) and a solution of carbonate **1** (3.9 g, 9.2 mmol) in dioxane (20 mL) was added. After 10 h at 80 °C the reaction mixture was evaporated under vacuum and the residue taken up in $CHCl_3$. The organic layer was washed with 0.5 N HCl, saturated aqueous Na_2CO_3 and H_2O, dried and concentrated and the residue eluted with $CHCl_3$:MeOH (95:5) from a silica gel column chromatography to give pure tripeptide *t*-butyl ester **4** as a foam (4.4 g, 81%).– $[\alpha]_D = +19.0°$ (c = 1, $CHCl_3$).– R_f 0.5 in $CHCl_3$/MeOH (97:3).– IR ($CHCl_3$): 3435, 3340, 1730-1660, 1495 cm^{-1}.– ^{1}H-NMR ([D_6]DMSO) δ = 1.4 (27H, s, 9 × CH_3), 1.85 (3H, s, CH_3CO), 2.6-2.9 (2H, m, Cys β-CH_2), 3.75 (2H, m, Acm CH_2), 4.0-4.3 (6H, m, Gly CH2, Cys α-CH, Glo α-CH and β-CH_2), 7.1 (1H, d, J = 7.0 Hz, Cys NH), 7.5 (1H, d, J = 9.0 Hz, Glo NH), 8.2 (1H, br. t, Acm NH), 8.5 (1H, br. t, Gly NH).– Anal. ($C_{25}H_{44}N_4O_{10}S$).

[Boc-Glo(-Cys-Gly-OBu^t)-OBu^t]$_2$ (**5**)

To a stirred solution of di-*t*-butyl ester **4** (4.0 g, 6.8 mmol) in MeOH (80 mL) I_2 (3.45 g, 13.6 mmol) in MeOH (40 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 partitioned 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 ($CHCl_3$:MeOH 97:3 as eluant) of the resulting crude product afforded pure disulfide ester **5** (2.4 g, 68%) as a white foam.– $[\alpha]_D = +26.0°$ (c = 1, $CHCl_3$).– R_f 0.7 in $CHCl_3$/MeOH (97:3).– IR ($CHCl_3$): 3420, 3340, 1740-1710, 1675, 1495 cm^{-1}.– ^{1}H-NMR ([D_6]DMSO) δ = 1.4 (54H, s, 18 × CH_3), 2.8 and 3.1 (4H, m, Cys β-CH_2), 3.7 (4H, m, Gly CH_2), 4.0–4.2 (6H, m, Glo α-CH and β-CH_2), 4.3 (2H, m, Cys α-CH), 7.1 (2H, d, J = 7.5 Hz, Glo NH), 7.55 (2H, d, J = 8.5 Hz, Cys NH), 8.3 (2H, t, J = 5.0 Hz, Gly NH).– Anal. ($C_{44}H_{76}N_6O_{18}S_2$).

[H-Glo(-Cys-Gly-OH)-OH]$_2$ (**6**)

The above described disulfide **5** (2.2 g, 2.1 mmol) was dissolved in TFA (40 mL) After 4 h at room temperature the solvent was removed and the residue repeatedly evaporated with ether to give 1.7 g (96%) of [TFA·H-Glo(-Cys-Gly-OH)-OH]$_2$ as a foam which was used without further purification.

The trifluoroacetate (1.5 g, 1.8 mmol) was dissolved in 1 N aqueous NH_3 (40 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 **6** as a white solid (1.0 g, 90%).– $[\alpha]_D = -63.0°$ (c = 1, H_2O).– R_f (D) 0.2 in *n*-BuOH/AcOH/H_2O (4:5:1).– IR (KBr) 3235 br., 1670-1550, 1395, 1255 cm^{-1}.– Anal. ($C_{18}H_{28}N_6O_{14}S_2$).

H-Glo(-Cys-Gly-OH)-OH (**7**)

A solution of the above reported disulfide **6** (1.0 g, 1.6 mmol) in a mixture of *n*-PrOH:H_2O (2:1) (30 mL) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. Tri-*n*-butylphosphine (0.4 g, 1.95 mmol) was added and the stoppered flask 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 by 1N HCl. Work up as described for disulfide **6** gave title compound **7** as a white solid (0.95 g, 96%).– $[\alpha]_D$ = –37.0° (c = 1, H_2O).– R_f 0.3 in *n*-BuOH/AcOH/H_2O (4:5:1).– IR (KBr) 3200 br., 1670-1555, 1400, 1260 cm^{-1}.– m/e (FAB, + ve ion) 310 $(m + 1)^+$.– Anal. ($C_9H_{15}N_3O_7S$).

Biological Assay

Hog kidney γ-glutamyltransferase (about 3 U/mg) was obtained from Fluka. GPNA and H-Gly-Gly-OH were purchased from Sigma. The concentration of compound **7**, after dissolution into 0.1 M Tris-HCl buffer, pH 8.0, was determined with Ellman's reagent [21]. *In vitro* assay of γ-glutamyltransferase activity was performed according to Meister *et al.* [22]

References

[1] V. J. Hruby, G. G. Bonner in *Methods in Molecular Biology*, Vol. 35: *Peptide Synthesis Protocols* (Eds.: M. W. Pennington, B. M. Dunn), Humana Press, Inc., Totowa, **1994**, 201-240.

[2] A. F. Spatola in *Methods in Neurosciences*, Vol. 13 (Ed.: P. M. Conn), Academic Press, Inc., San Diego, **1993**, 19–42.

[3] K. T. Douglas in *Offprints from Glutathione: Chemical, Biochemical and Medical Aspects* (Eds.: D. Dolphin, R. Poulson, D. Avramovic), Wiley, New York, **1986**, 243–279.

[4] H. E. Witkowska, C. Wasielewski, *Int. J. Peptide Protein Res.* **1989**, *33*, 154–161.

[5] L. Sheh, B. L. Chen, C. F. Chen, *Int. J. Peptide Protein Res.* **1990**, *35*, 55–62.

[6] M. H. Lyttle, A. Satyam, M. D. Hocker, K. E. Bauer, C. G. Caldwell, H. C. Hui, A. S. Morgan, A. Mergia, L. M. Kauvar, *J. Med. Chem.* **1994**, *37*, 1501–1507.

[7] W. J. Chen, D. Y. Lee, R. N. Armstrong, *J. Org. Chem.* **1986**, *51*, 2848–2850.

[8] G. Luisi, A. Calcagni, F. Pinnen, *Tetrahedron Lett.* **1993**, *34*, 2391–2392.

[9] A. Calcagni, S. Dupré, G. Lucente, G. Luisi, F. Pinnen, D. Rossi, *Int. J. Peptide Protein Res.*, **1995**, *46*, 434–439.

[10] M. Y. Gololobov, R. C. Bateman, Jr., *Biochem. J.* **1994**, *304*, 869–876.

[11] Y. Ikeda, J. Fujii, M. E. Anderson, N. Taniguchi, A. Meister, *J. Biol. Chem.* **1995**, *270*, 22223–22228.

[12] A. Meister, S. S. Tate, *Annu. Rev. Biochem.* **1976**, *45*, 559–604.

[13] M. Orlowski, A. Meister, *Biochim. Biophys. Acta* **1963**, *73*, 679–681.

[14] S. S. Tate, A. Meister, *J. Biol. Chem.* **1974**, *249*, 7593–7602.

[15] B. Kamber, *Helv. Chim. Acta.* **1971**, *54*, 927–930.

[16] G. Jung, E. Breitmaier, W. Voelter, *Eur. J. Biochem.* **1972**, *24*, 438–445.

[17] E. Benedetti, C. Pedone, C. Toniolo, G. Némethy, M. S. Pottle, H. A. Scheraga, *Int. J. Peptide Protein Res.* **1980**, *16*, 156–172.

[18] E. Benedetti, C. Pedone, C. Toniolo, M. Dudek, G. Némethy, H. A. Scheraga, *Int. J. Peptide Protein Res.* **1983**, *21*, 163–181.

[19] Y. Wu, J. Kohn, *J. Am. Chem. Soc.* **1991**, *113*, 687–688.

[20] A. Bax, G. A. Morris, *J. Magn. Reson.* **1981**, *42*, 501–505.

[21] G. L. Ellman, *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.

[22] A. Meister, S. S. Tate, O. W. Griffith in *Methods in Enzymology*, Vol. 77 (Ed. W. B. Jakoby), Academic Press, New York, **1981**, 237–242.

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