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# Synthesis and activity of novel glutathione analogues containing an urethane backbone linkage

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Dedicated to the memory of Professor Piero Pratesi

**Abstract**

The new GSH analogues H–Glo(–Ser–Gly–OH)–OH (**5**), its *O*-benzyl derivative **4**, and H–Glo(–Asp–Gly–OH)–OH (**9**), characterized by the replacement of central cysteine with either serine or aspartic acid, and containing an urethanic fragment as isosteric substitution of the scissile γ-glutamylic junction, have been synthesized and characterized. Their ability to inhibit human GST P1-1 (hGST P1-1) in comparison with H–Glu(–Ser–Gly–OH)–OH and H–Glu(–Asp–Gly–OH)–OH, which are potent competitive inhibitors of rat GST 3-3 and 4-4, has been evaluated. In order to further investigate the effect of the isosteric substitution on the binding abilities of the new GSH analogues **4**, **5** and **9**, the previously reported cysteinyl-containing analogue H–Glo(–Cys–Gly–OH)–OH has been also evaluated as a co-substrate for hGSTP1-1.


*Keywords:* γ-Glutamyl junction; γ-Glutamyl transpeptidase; Glutathione; Glutathione analogues; Human glutathione *S*-transferase; Urethanic bond

## 1. Introduction

A major pathway in the cellular (de)toxification of both xenobiotic and endobiotic electrophiles is represented by conjugation with tripeptide glutathione (γ-Glu–Cys–Gly, GSH). This reaction is catalysed by the isoenzyme family of glutathione *S*-transferases (GSTs, E.C. 2.5.1.18) [1,2], which are mainly present in high concentration in the cytosol of many tissues. Based on sequence similarity, immunological cross-reactivity, and subcellular distribution, cytosolic GSTs have been divided into at least ten gene-independent classes (Alpha, Beta, Delta, Kappa, Pi, Mu, Sigma, Theta, Zeta and Omega), which show different but overlapping substrate specificities, while sharing, not surprisingly, a high specificity for the physiologic co-substrate GSH [3]. The suggestion that GSH conjugation may be concerned with anticancer drug resistance has evoked additional interest in this group of enzymes [4,5]. Although it is not clear whether GST overexpression is the result or a cause of resistance to chemotherapy, the approach of GST inhibition with the aim of providing tumor-directed potentiation of conventional antineoplastic agents appears challenging. In this context the human Pi-class GST (hGST P1-1) represents an attractive target, since it is the predominant isoform in most tumors [6,7].

*Abbreviations:* AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; *n*-BuOH, *n*-butanolo; Bzl, benzyl; CDNB, 1-chloro-2,4-dinitrobenzene; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; EDTA, ethylenediamine tetraacetic acid; $Et_2O$, diethyl ether; EtOAc, ethyl acetate; Fmoc, 9-fluorenylmethoxycarbonyl; Glo, L-γ-oxaglutamic acid; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Z, benzyloxycarbonyl.

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Structural and functional aspects concerning GSTs are the subject of extensive studies [1,8–11]; much interest is focused on the one part of the protein which is essentially conserved among all the GSTs, the GSH-binding site (G-site); here the co-substrate is accomodated, adopting the proper alignment at the reaction site prior to thiol deprotonation.

Thus several GSH analogues and derivatives, including a variety of γ-glutamyl modifications, have been synthesized in an attempt to design specific GST inhibitors and/or as probes for the G-site in GSTs [12,13]. However, GST inhibitors retaining the GSH isopeptidic structure are expected to be ineffective in vivo, as they are still processed by γ-glutamyl transpeptidase (γ-GT), a cell-surface enzyme that initiates the breakdown of GSH and its *S*-substituted derivatives at the level of the γ-glutamyl junction [14,15]. Interestingly, with very few notable exceptions [16] the majority of the chemical variations reported so far does not involve the GSH γ-glutamylic junction, in spite of the relevant consequences this approach is expected to introduce in terms of resistance to γ-GT.

As a prosecution of our studies on the structural modification of GSH [17–22], we started a research program aimed at studying GSH analogues characterized by two fundamental properties: (i) close resemblance with the natural substrate as to overall peptidic structure, molecular size, and functional group arrangement; and (ii) altered structure and reactivity of the γ-glutamylic CO–NH junction obtained through chemical modification or isosteric 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 the enzymatic system involved. 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. These modified compounds may behave as substrates, often with quite different kinetics constants, or inhibitors, often of competitive nature if the chemical modifications do not alter dramatically the spatial structure.

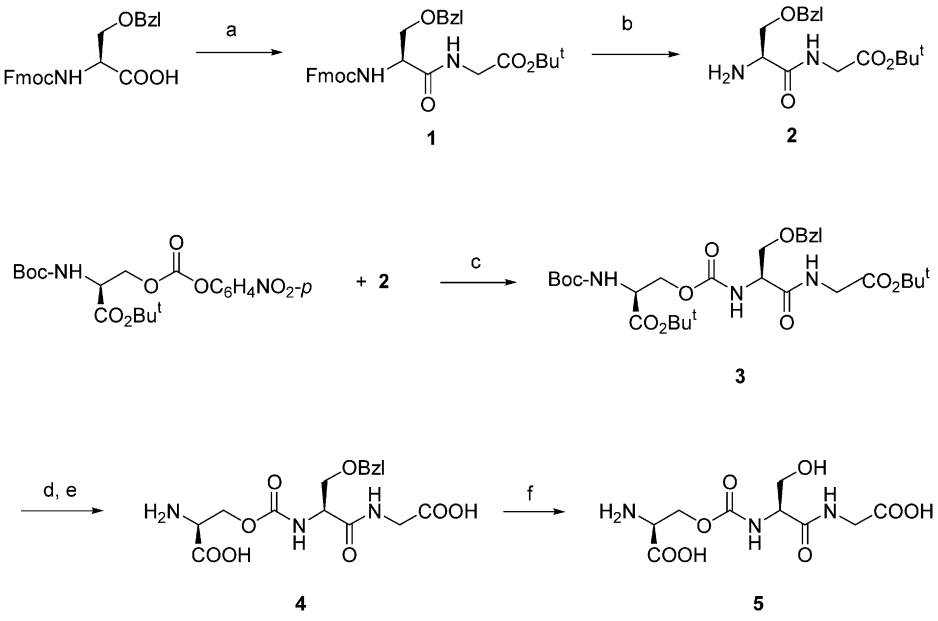
This chemical modification has been recently introduced by us with the synthesis of backbone-modified GSH analogues, obtained through replacement of the Glu γ-carbon atom adjacent to the isopeptidic bond with an aza (NH) and an oxa (O) unit [20,21]. The resulting pseudopeptides, characterized respectively by the presence of ureic (NHCONH) and urethanic (OCONH) junctions as γ-glutamylic bond surrogates, were found stable γ-GT inhibitors [2,19–21]. This original approach has been subsequently adopted by other Authors for the synthesis of metabolically stable inhibitors of glyoxalase I [23] and novel γ-GT resistant GST inhibitors [24].

As an extension of our research in this field, we become interested in developing peptidic GSH analogues stabilized against γ-GT degradation, to be tested as potential hGST P1-1 inhibitors. The GSH-related pseudopeptides considered in this study are characterized by the replacement of central cysteine with serine ed aspartic acid, respectively, and contain an urethanic fragment as isosteric substitution of the scissile glutamylic junction, which is expected to confer the desired metabolic stability; thus, from a formal point of view, the designed analogues contain the L-γ-oxaglutamic acid (Glo) replacing the Glu residue. The replacement of cysteine thiol with more electronegative groups such as carboxylates or hydroxyls (Ser or Asp) is a well-documented strategy to potent GST competitive inhibitors at the GSH binding site [25,26]. On the other hand, urethane bonds appear to be promising peptide bond surrogates due to structure planarity, hydrogen-bonding characteristics and defined conformational features. Unlike the majority of peptide bond surrogates, which mimic the two atom sequence of conventional peptide unit, the urethane bond consists of an extended triatomic (O–C–N) planar system, made of an ester and an amide portion, suitable to introduce unique conformational and electronic properties at the backbone level [27,28]. We report here synthesis, characterization and biological activity of novel GSH-related compounds, the two oxy-analogues H–Glo(–Ser–Gly–OH)–OH (5) and its *O*-benzyl derivative H–Glo[–Ser(Bzl)–Gly–OH]–OH (4), and H–Glo(–Asp–Gly–OH)–OH (9) (Schemes 1 and 2). The analogue **4**, featuring a $PhCH_2$ appendix at the central residue, appears as a suitable model to explore the hydrophobic requirements of the adduct-binding site.

## 2. Experimental

### 2.1. Peptide synthesis

Melting points were determined on a Büchi B-450 apparatus and are uncorrected. TLC was performed on Merck 60 $F_{254}$ silica gel plates developed with the following solvent system: (a) $CHCl_3$:MeOH (98:2); (b) $CHCl_3$:MeOH (96:4); (c) $CHCl_3$:$Et_2O$ (1:1); (d) *n*-BuOH:AcOH:$H_2O$ (4:5:1); (e) *n*-BuOH:AcOH:$H_2O$ (6:4:4), (f) $CHCl_3$:MeOH (99:1). Column chromathography was carried out using Merck 60 silica gel (230–400 mesh). Optical rotations were taken at 20 °C with a Perkin–Elmer 241 polarimeter. IR spectra were recorded employing a Perkin–Elmer 983 FTIR-1600

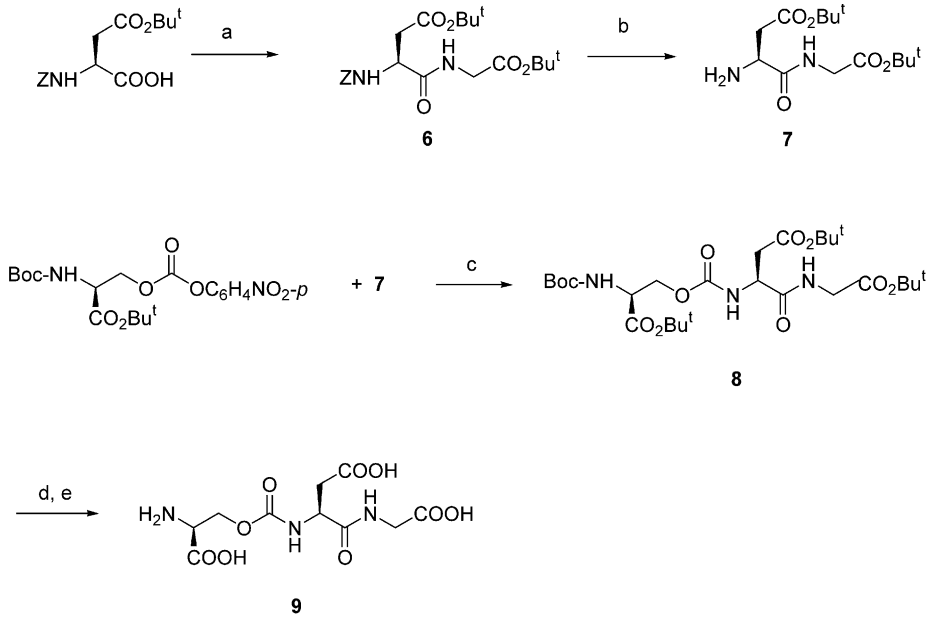



Scheme 1. Reagents and conditions. (a) HCl·H–OBu$^t$, HOBt, NMM, DCCl, THF, 0 °C, 4 h, then 5 °C, 16 h; (b) DBU, $CH_2Cl_2$, r.t., 20 min; (c) dioxane, 80 °C, 10 h; (d) TFA, r.t., 5 h; (e) 1 N aq. $NH_3$, r.t., 30 min; (f) $H_2$, 10% Pd–C, AcOH, r.t., 6 h.

spectrophotometer. $^1$H and $^{13}$C NMR spectra were determined on a Varian VXR 300 MHz instrument ($\delta$ expressed in ppm). NMR resonances of analogues **4**, **5** and **9** were assigned with standard 2D NMR techniques (HETCOR). Elemental analyses for the new compounds are within the $\pm$0.4% of the theoretical values. Fmoc–Ser(Bzl)–OH and Z–Asp(OBu$^t$)–OH were obtained from Novabiochem. HCl·H–Gly–OBu$^t$ was purchased from Sigma Aldrich.

*2.1.1. Fmoc–Ser(Bzl)–Gly–OBu$^t$ (**1**)*

Fmoc–Ser(Bzl)–OH (2 g, 4.8 mmol) was dissolved in THF (8 ml) and HOBt (0.65 g, 4.8 mmol) was added with stirring. The solution was cooled to 0 °C and an ice–cold solution containing HCl·H–Gly–OBu$^t$ (0.8 g, 4.8 mmol) in THF (8 ml) was added, followed by portionwise addition of a solution of DCC (0.99 g, 4.8 mmol) in THF (1 ml). After 4 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting




Scheme 2. Reagents and conditions. (a) HCl·H–Gly–OBu$^t$, HOBt, NMM, DCCl, THF, 0 °C, 4 h, then 5 °C, 16 h; (b) $H_2$, 10% Pd–C, MeOH, r.t., 2 h; (c) dioxane, 80 °C, 10 h; (d) TFA, r.t., 5 h; (e) 1 N aq. $NH_3$, r.t., 30 min.

solution was evaporated under vacuum. The residue was taken up in EtOAc and the organic layer washed with 1 N $KHSO_4$, saturated acqueous $NaHCO_3$ and $H_2O$. The residue obtained after drying and evaporation was cromatographed on silica gel using $CHCl_3$:MeOH (99:1) as eluant to give dipeptide *t*-butyl ester **1** as a white solid, further purified by crystallization from EtOAc (2.4 g, 94%). m.p. 128–9 °C. $R_f$ (a) = 0.7. $[\alpha]_{546} = +3.0^\circ$ ($c = 1$, $CHCl_3$). IR (KBr) 3405, 3250, 1725, 1655, 1535 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) $\delta = 1.5$ (s, 9H, $3 \times CH_3$), 3.6 (m, 1H, Ser β-$CH_B$), 3.9 (m, 3H, Gly $CH_2$ and Ser α-CH), 4.2 (m, 1H, Fmoc CH), 4.4 (m, 3H, Fmoc $OCH_2$ and Ser β-$CH_A$), 4.6 (m, 2H, $PhCH_2O$), 5.7 (br d, 1H, Ser NH), 6.95 (br t, 1H, Gly NH), 7.2–7.4 (m, 9H, ArH), 7.6 (2d overlapped, 2H, ArH) and 7.8 (2d overlapped, 2H, ArH).

### 2.1.2. *H–Ser(Bzl)–Gly–OBu$^t$ (**2**)*

To a solution of the above reported *t*-butyl ester **1** (2.3 g, 4.35 mmol) in $CH_2Cl_2$ (30 ml) DBU (0.69 g, 4.36 mmol) was added at room temperature. After 20 min the solution was evaporated to dryness and the residue chromatographed on silica gel using $CHCl_3$:MeOH (95:5) as eluant to yield pure *N*-deprotected dipeptide ester **2** as an oil (1.32 g, 98%). $R_f$ (b) = 0.3. $[\alpha]_D = -38.0^\circ$ ($c = 1$, $CHCl_3$). IR ($CHCl_3$) 3370, 1735, 1670 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) $\delta = 1.5$ (s, 9H, $3 \times CH_3$), 1.8 (s, 2H, Ser $NH_2$), 3.6–3.75 (m, 3H, Ser α-CH and β-$CH_2$), 3.9 (ABq, $J = 10$ Hz, 2H, Gly $CH_2$), 4.55 (s, 2H, $PhCH_2O$), 7.3 (m, 5H, ArH), 7.9 (br t, 1H, Gly NH).

### 2.1.3. *Boc–Glo[–Ser(Bzl)–Gly–OBu$^t$]–OBu$^t$ (**3**)*

The above described dipeptide *t*-butyl ester **2** (1.3 g, 4.25 mmol) was dissolved in dioxane (10 ml) and a solution of Boc–Glo(ON*p*)–OBu$^t$ [21] (1.8 g, 4.25 mmol) in dioxane (10 ml) was added. After 10 h at 80 °C the reaction mixture was evaporated under reduced pressure and the residue taken up in $CHCl_3$. The organic layer was washed with 0.5 N HCl, saturated acqueous $Na_2CO_3$ and $H_2O$. The residue obtained after drying and evaporation was cromatographed on silica gel using $CHCl_3$:MeOH (99:1) as eluant to give tripeptide *t*-butyl ester **3** as a foam (1.97 g, 78%). $R_f$ (c) = 0.6; $[\alpha]_D = +24^\circ$ ($c = 1$), $CHCl_3$). IR ($CHCl_3$): 3425, 1735–1700, 1680, 1500 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) $\delta = 1.45$ (s, 27H, $9 \times CH_3$), 3.95 (d, 2H, Gly $CH_2$), 4.3–4.5 (m, 6H, Ser and Glo α-CH and β-$CH_2$), 4.6 (ABq, $J = 12.0$ Hz, 2H, $PhCH_2O$), 5.4 (d, $J = 4.8$ Hz, 1H, Glo NH), 5.7 (br d, 1H, Ser NH), 7.0 (t, $J = 4.5$ Hz, 1H, Gly NH), 7.4 (m, 5H, ArH).

### 2.1.4. *H–Glo[–Ser(Bzl)–Gly–OH]–OH (**4**)*

The preceding foam **3** (1.55 g, 2.6 mmol) was treated with TFA (40 ml) at room temperature. After 5 h the solvent was removed and the residue repeatedly evaporated with ether to give 0.94 g (73%) of TFA·H–Glo[–Ser(Bzl)–Gly–OH]–OH, which was used without further purification.

The trifluoroacetate (0.94 g, 1.89 mmol) was dissolved in 1 N aqueous $NH_3$ (14.6 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 eluant to afford tripeptide acid **4** as a foam (0.67 g, 92%). $R_f$ (d) = 0.85. $[\alpha]_D = -3.0^\circ$ ($c = 1$, $H_2O$). IR (KBr) 3255 br, 1710, 1670–1550, 1395, 1250 $cm^{-1}$. $^1H$ NMR ($D_2O$) $\delta = 3.7$ (m, 2H, Ser β-$CH_2$), 3.75 (app s, 2H, Gly $CH_2$), 3.95 (m, 1H, Glo α-CH), 4.35 (m, 1H, Ser α-CH), 4.4 (m, 2H, Glo $CH_2$), 4.45 (m, 2H, $PhCH_2O$), 7.3 (m, 5H, ArH). $^{13}C$ NMR ($D_2O$) $\delta = 46.00$ (Gly $C^\alpha$), 56.80 (Glo $C^\alpha$), 57.91 (Ser $C^\alpha$), 66.65 (Glo $C^\beta$), 71.85 (Ser $C^\beta$), 75.84 ($PhCH_2O$), 131.21, 131.25, 131.57 and 139.87 (aromatics), 159.79 (Glo OCO), 173.88, 174.30 and 178.66 (CO).

### 2.1.5. *H–Glo(–Ser–Gly–OH)–OH (**5**)*

A solution of the *O*-benzyl derivative **4** (0.65 g, 1.7 mmol) in glacial AcOH (42 ml) was hydrogenated in the presence of 10% Pd on activated charcoal (0.25 g). After 6 h the catalyst was filtered off and the filtrate was evaporated under vacuum. The residue was chromathograped on Sephadex LH-20 using $H_2O$:MeOH (2:1) as eluant to yield title compound **5** as a white solid (0.35 g, 70%). $R_f$ (e) = 0.25; $[\alpha]_D = -7.0^\circ$ ($c = 1$, $H_2O$). IR (KBr) 3235 br, 1670–1555, 1400, 1255 $cm^{-1}$.

### 2.1.6. *Z–Asp(OBu$^t$)–Gly–OBu$^t$ (**6**)*

This compound was prepared by following the procedure reported for the *N*-protected dipeptide *t*-butyl ester **1**, starting from HCl·H–Gly–OBu$^t$ (1.5 g, 8.8 mmol) and Z–Asp(OBu$^t$)–OH (3.0 g, 8.8 mmol). The resulting residue was purified by column chromatography ($CHCl_3$: MeOH 99:1 as eluant) to give the dipeptide *t*-butyl ester **6** as an oil (3.38 g, 88%). $R_f$ (f) = 0.50. $[\alpha]_D = +15.5^\circ$ ($c = 1$, $CHCl_3$). IR (KBr) 3410, 3360, 2980, 1725, 1655, 1530 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) $\delta = 1.5$ (s, 18H, $6 \times CH_3$), 2.65 (m, 1H, Asp β-$CH_B$), 2.9 (m, 1H, Asp β-$CH_A$), 3.9 (m, 2H, Gly $CH_2$), 4.6 (m, 1H, Asp α-CH), 5.15 (m, 2H, $PhCH_2O$), 6.0 (d, $J = 7.92$ Hz, 1H, Asp NH), 6.95 (br t, 1H, Gly NH), 7.2–7.4 (m, 5H, ArH).

### 2.1.7. *H–Asp(OBu$^t$)–Gly–OBu$^t$ (**7**)*

A solution of the *N*-benzyloxycarbonyl derivative **6** (3.2 g, 7.3 mmol) in MeOH (360 ml) was hydrogenated in the presence of 10% Pd on activated charcoal (2.1 g). After 2 h the catalyst was removed by filtration and the solution evaporated under reduced pressure to afford the pure *N*-deprotected dipeptide ester **7** as an oil (2.14 g, 97%). $R_f$ (f) = 0.25. $[\alpha]_D = -14.5^\circ$ ($c = 1$, $CHCl_3$). IR (neat): 3380, 3005, 2980, 1725, 1675, 1520, 1455 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) $\delta = 1.5$ (s, 18H, $6 \times CH_3$), 1.8 (s, 2H, Asp $NH_2$), 2.51 (m, 1H, Asp β-$CH_B$), 2.82 (m, 1H, Asp

β-$CH_A$), 3.68 (m, 1H, Asp α-CH), 3.9 (m, 2H, Gly $CH_2$), 7.83 (br t, 1H, Gly NH).

*2.1.8. Boc–Glo[–Asp(OBu$^t$)–Gly–OBu$^t$]–OBu$^t$ (**8**)*

To a solution of the foregoing dipeptide *t*-butyl ester **7** (2.0 g, 6.6 mmol) in DMF (20 ml) a solution of Boc–Glo(ON*p*)–OBu$^t$ (2.81 g, 6.6 mmol) in DMF (20 ml) was added. After 10 h at 80 °C the reaction mixture was evaporated under vacuum and the residue partitioned between EtOAc and $H_2O$. The organic layer was washed with 1 N $KHSO_4$, saturated acqueous $Na_2CO_3$ and $H_2O$. The residue obtained after drying and evaporation was cromatographed on silica gel using $CHCl_3$:MeOH (99:1) as eluant to give tripeptide *t*-butyl ester **8** as a foam (2.1 g, 54%). $R_f$ (f) = 0.55. $[\alpha]_D = +12.8°$ ($c = 1$, $CHCl_3$). IR (neat): 3345, 2980, 2930, 1730, 1520, 1370 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) $\delta = 1.43$ (s, 36H, $12 \times CH_3$), 2.63 (m, 1H, Asp β-$CH_B$), 2.90 (m, 1H, Asp β-$CH_A$), 3.89 (m, 2H, Gly $CH_2$), 4.30–4.54 (m, 4H, Asp α-CH, Glo α-CH and Glo β-$CH_2$), 5.35 (d, $J = 6.6$ Hz, 1H, Glo NH), 5.96 (d, $J = 7.91$ Hz, 1H, Asp NH), 6.96 (t, $J = 6.18$ Hz, 1H, Gly NH).

*2.1.9. H–Glo[–Asp(OH)–Gly–OH]–OH (**9**)*

The above described tripeptide **8** (2.0 g, 3.4 mmol) was dissolved in TFA (10 ml). After 5 h at room temperature the solvent was removed and the residue repeatedly evaporated with ether to give TFA·H–Glo(–Asp–Gly–OH)–OH in quantitative yield, which was used as such.

The trifluoroacetate (1.9 g, 4.3 mmol) was dissolved in 1 N aqueous $NH_3$ (100 ml) at room temperature. After 30 min the aqueous solution was concentrated and subjected to column chromatography on Sephadex LH-20 using $H_2O$:MeOH (2:1) as eluant to afford **9** as a white solid (1.15 g, 83%). $R_f$ (d) = 0.45. $[\alpha]_D = +19.4°$ ($c = 1$, $H_2O$). IR (neat): 3135 br, 2360, 1705, 1650, 1400 $cm^{-1}$.

### 2.2. Biological assays

Inhibition experiments *in vitro* concerning compounds **4**, **5** and **9** were performed on human isoenzyme GST P1-1 (EC 2.5.1.18), using CDNB as acceptor substrate to characterize GSH conjugation. Human placenta GST P1-1 was expressed in *Escherichia coli* and purified as previously described [29]. GSTP1-1 activity was measured at 25 °C in 1 ml of 0.1 M K-phosphate buffer pH 6.5 containing 1 mM GSH {or 1 mM H–Glo(–Cys–Gly–OH)–OH [21]}, 1 mM CDNB and 0.1 mM EDTA. GSTP1-1 inhibition experiments were performed in the presence of variable amounts of GSH analogues, ranging from 0.01 to 8 mM for H–Glo[–Ser(Bzl)–Gly–OH]–OH (4), H–Glo(–Ser–Gly–OH)–OH (5), and H–Glu(–Ser–Gly–OH)–OH (GOH), and from 0.01 to 2 mM for H–Glo(–Asp–Gly–OH)–OH (**9**) and H–Glu(–Asp–Gly–OH)–OH. Activity was recorded at 340 nm ($\varepsilon = 9.6$ $mM^{-1}$ $cm^{-1}$), with a Kontron double-beam Uvikon 940 spectrophotometer and normalized for the rate of reaction obtained in the absence of inhibitor.

## 3. Results and discussion

### 3.1. Chemical synthesis

The GSH analogues **4**, **5** and **9** were synthesized in good overall yields using solution procedures by stepwise elongation of the peptide chain in the *C*-to-*N* direction. The common adopted synthetic strategy, which provides for the introduction of the urethane OCONH linkage in place of the γ-glutamyl $CH_2$–CO–NH fragment, is based on the utilization of an *O*-functionalized serine residue as building block of the modified γ-glutamyl moiety. Thus, the active carbonate Boc–Glo(ON*p*)–OBu$^t$ was synthesized starting from Boc–Ser–OBu$^t$ and *p*-nitrophenyl chloroformate, as described elsewhere [21], to be condensed with the amino group of the protected dipeptides.

The route towards the γ-glutamyl modified oxy-analogues **4** and **5** is outlined in Scheme 1. Dipeptide amide H–Ser(Bzl)–Gly–OBu$^t$ (**2**) was prepared by conventional DCC/HOBt condensation of Fmoc–Ser(Bzl)–OH with H–Gly–OBu$^t$ and subsequent removal of the Fmoc protecting group with DBU at r.t. [18]. The *N*-deprotected dipeptide **2** was then coupled with Boc–Glo(ON*p*)–OBu$^t$ in dioxane at 80 °C for 10 h to give the fully protected tripeptide Boc–Glo[–Ser(Bzl)–Gly–OBu$^t$]–OBu$^t$ (**3**) in 80% yield. Subsequent removal in a single step of the *tert*-butyl and *tert*-butyloxycarbonyl protecting groups by using TFA, followed by treatment of the resulting trifluoroacetate with aqueous ammonia, afforded H-Glo[–Ser(Bzl)–Gly–OH]–OH (4) in 70% yields. The *O*-benzyl derivative **4** was then converted by catalytic hydrogenolysis with 10% Pd–C in AcOH into the GSH oxy-analogue H–Glo(–Ser–Gly–OH)–OH (**5**), without any side reactions and in good yields.

Accordingly, synthesis of compound **9** was straightforward (see Scheme 2), commencing with the DCC/HOBt mediated acylation of Z–Asp(OBu$^t$)–OH with H–Gly–OBu$^t$ to give the protected dipeptide **6**. The amino functionality was recovered by catalytic hydrogenolysis and coupled to activated *tert*-butyloxycarbonyl-L-γ-oxaglutamic acid under the same conditions reported for dipeptide **2**, to give acceptable amounts of the protected tripeptide Boc–Glo[–Asp(OBu$^t$)–Gly–OBu$^t$]–OBu$^t$ (**8**). In analogy with the oxy-compound 3, complete deprotection was easily performed by acidolysis with TFA, followed by addition of aqueous ammonia to ensure the conversion into the free amino

tripeptide acid H–Glo(–Asp–Gly–OH)–OH (**9**) in good yields.

The final peptides were purified to apparent homogeneity by gel-permeation chromatography and fully characterized by $^{1}H$ and $^{13}C$ NMR spectroscopy (see Table 1).

### 3.2. Biological studies

The new GSH analogues H–Glo(–Ser–Gly–OH)–OH (**5**), its *O*-benzyl derivative **4**, and H–Glo(–Asp–Gly–OH)–OH (**9**) were tested for their ability to inhibit human GST P1-1 (hGST P1-1) in comparison with H–Glu(–Ser–Gly–OH)–OH (GOH) and H–Glu(–Asp–Gly–OH)–OH, which are potent competitive inhibitors of rat GST 3-3 and 4-4, as previously reported [25,26]. Fig. 1 shows the effect on the enzymatic activity of the three GSH oxy-analogues **4**, **5**, and GOH (panel A), and of the two aspartyl–peptides **9** and H–Glu(–Asp–Gly–OH)–OH (panel B). Compounds **4**, **5** and **9** are not inhibitors of hGST P1-1 while, in the same assay conditions, GOH and H–Glu(–Asp–Gly–OH)–OH show $IC_{50}$ values of $0.79 \pm 0.04$ and $0.05 \pm 0.01$ mM, respectively. Assuming for these compounds a competitive inhibition towards GSH $\{K_m = 0.22$ mM [30]$\}$, the $IC_{50}$ values correspond to $K_i$ of 142 and 9 μM, respectively. The present data indicate that GOH and H–Glu(–Asp–Gly–OH)–OH can inhibit even the human GST P1-1 isoform, although ten times less than rat liver GST 3-3 and 4-4.

In order to further investigate the effect of the isosteric substitution on the binding abilities of the new GSH analogues **4**, **5** and **9**, we tested the previously synthesized cysteinyl-containing pseudopeptide H–Glo(–Cys–Gly–OH)–OH [21] as a co-substrate for hGSTP1-1. The reactivity of this GSH analogue is quite low, as the isosteric substitution resulted in a fivefold decrease of catalytic efficiency compared to that obtained with GSH.

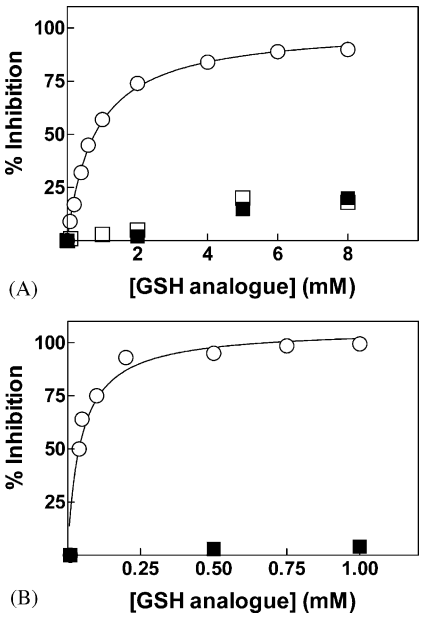



Fig. 1. Inhibition of human placenta GSTP1-1 by GSH analogues. GST activity was measured at 25 °C in the presence of 1 mM GSH and 1 mM CDNB, as described under Section 2. Panel A: H–Glu(–Ser–Gly–OH)–OH (GOH) (○), H–Glo[–Ser(Bzl)–Gly–OH]–OH (**4**) (■), H–Glo(–Ser–Gly–OH)–OH (**5**) (□). The solid line is the best fit of the experimental data to a hyperbolic binding equation which fulfils an $IC_{50} = 0.79 \pm 0.04$ mM. Assuming a competitive inhibition vs. GSH, a $K_i = 142$ μM can be calculated for the inhibitor GOH. Panel B: H–Glu(–Asp–Gly–OH)–OH (○), H–Glo(–Asp–Gly–OH)–OH (9) (■). The solid line is the best fit of the experimental data to a hyperbolic binding equation which fulfils an $IC_{50} = 0.05 \pm 0.01$ mM. Assuming a competitive inhibition vs. GSH, a $K_i = 9$ μM can be calculated for the inhibitor H–Glu(–Asp–Gly–OH)–OH. Data shown in the figure represent the mean of three different experiments; the standard error for each point does not exceed 5%.

Table 1
$^{1}H$ and $^{13}C$ NMR data (in $D_2O$ at 25 °C) for compounds **5** and **9**

| Residue | H—Glo(–Ser–Gly–OH)–OH (**5**) | | H–Glo-(–Asp–Gly–OH)–OH (**9**) | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| *Glo* | | | | |
| $C^{\alpha}$ | 4.0 | 57.53 | 3.85 | 53.98 |
| $C^{\beta}$ | 4.45 | 66.63 | 4.2–4.45 | 63.80 |
| CO–O | | 174.83 [a] | | 173.57 [b] |
| O–CO–N | | 159.91 | | 157.01 |
| *Ser or Asp* | | | | |
| $C^{\alpha}$ | 4.2 | 59.81 | 4.3 | 52.76 [b] |
| $C^{\beta}$ | 3.75 | 64.27 | 2.45 ($H_B$), 2.6 ($H_A$) | 38.13 |
| CO–N | | 170.58 | | 171.29 [b] |
| CO–O | | | | 177.00 |
| *Gly* | | | | |
| $C^{\alpha}$ | 3.7 | 46.20 | 3.6 | 43.12 |
| CO | | 174.91 [a] | | 176.04 |

[a] Assignments may be interchanged.
[b] Assignments may be interchanged.

## 4. Conclusions

Results from the inhibition studies on hGST P1-1 show that the tested compounds **4**, **5** and **9** are devoid of activity, although they meet some requirements for good in vivo GST inhibitors. In fact all the pseudopeptides have been designed to be γ-GT resistant, through isosteric replacement of the γ-glutamylic isopeptidic bond with an urethane moiety [21]. Furthermore they are modified at the central residue with the introduction of L-amino acids featuring electronegative groups at the $C^{\beta}$ atom; the exploitment of this last approach has previously led to potent GST 3-3 and 4-4 inhibitors. In

this regard it is interesting to note that also the isoenzyme of the Pi-class is inhibited by the two reported inhibitors H–Glu(–Ser–Gly–OH)–OH (GOH) and H–Glu(–Asp–Gly–OH)–OH, however with at least one order of magnitude less affinity. These data are consistent with slight variations in the G-site structure among these isoenzymic classes, which result in different hydrogen-bonding co-substrate interactions involving protein residues other than the conserved ones.

These results suggest that substitution of the oxygen for the γ-$CH_2$ bound to the γ-Glu amide carbonyl is responsible for a non productive binding of the GSH analogues to the G-site, confirming that the γ-glutamyl moiety is the major binding determinant in protein-ligand multiple interaction pattern. Accordingly, the cysteinyl-containing isostere shows a poor propensity to act as a co-substrate in the GST-mediated conjugation with CDNB.

The lack of inhibitory activity observed for the new compounds may be explained in terms of both conformational and electronic consequences of the isosteric replacement. Several conformational investigations on this peptide bond surrogate are centered on *N*-terminal protected *tert*-butoxycarbonyl- and benzyloxycarbonyl-amino acids and peptides [27,28,31]. A notable exception is represented by the work of Parkinson et al., who reported in detail the conformational properties of urethane linkages when inserted into the peptide backbone [32]. They evidenced the tendency of this moiety to adopt a *trans–trans* conformation at the level of the ester and amide portions of the extended planar system, respectively; in this particular arrangement the O–CO fragment features the pseudopeptide oxygen in place of the peptide $C^{\alpha}$ atom and its lone pairs at the position of the $C^{\beta}$ group. The lack of activity in the tested compounds should be ascribed to alterations in enzyme interactions connected with the specific conformational properties of the urethane bond. However, the likely occurrence of alternative conformations at the modified γ-Glu linkage is not the only explanation for inactivity; the electronic properties as well as the different H-bonding patterns of the urethane junction may be as well of significance in modulating the interactions in the protein active sites.

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