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Peptidomimetic Glutathione Analogues as Novel γGT Stable GST Inhibitors

Danny Burg,[a,*] Dmitri V. Filippov,[b] Ralph Hermanns,[a] Gijs A. van der Marel,[b] Jacques H. van Boom[b] and Gerard J. Mulder[a]

[a]*Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, PO Box 9503, 2300RA, Leiden, The Netherlands*

[b]*Division of Bio-Organic Synthesis, Leiden Institute of Chemistry, PO Box 9502, 2300RA, Leiden University, The Netherlands*

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Abstract—Elevated levels of glutathione-*S*-transferase (GST) isoenzymes are found in many tumor cells and are thought to play a role in the onset of multidrug resistance (MDR). To evaluate the contribution of GST to this process, inhibitors are needed. Glutathione (GSH) conjugates, although good GST inhibitors, cannot be used in vivo, because they are eliminated rapidly. In this paper, we describe the synthesis of a series of novel peptidomimetic glutathione analogues that are stabilized against peptidase mediated breakdown. The peptide bonds in GSH were replaced by isosteres, such as the 'reduced' amide (which was prepared using a novel method), *N*-methylamide, urethane, and methylene linkages. The in vitro evaluation of the compounds focuses on GST inhibition and stability towards γ-glutamyl-transpeptidase (γGT), the main enzyme involved in GSH breakdown. The compounds were conjugated to the model electrophile ethacrynic acid (EA) to resemble GS-EA, an efficient GST inhibitor. All novel GSH-analogues were shown to inhibit rat liver cytosolic GSTs. Furthermore, peptidomimetic changes of the γ-glutamyl-cysteine amide bond greatly improved stability towards γGT. These compounds may therefore be useful in the design of novel in vivo applicable GST inhibitors.

Introduction

The ubiquitous tripeptide glutathione (GSH), γ-L-glutamyl-L-cysteinyl-glycine, is the prevalent non-protein thiol in eukaryotic cells. GSH plays a critical role in the cellular protection against potentially harmful electrophiles from xenobiotic sources, or those generated by endogenous oxidative processes.[1] GSH-conjugates are formed by nucleophilic attack of the cysteine sulphydryl on the electrophilic center of a suitable substrate. This process is catalyzed by glutathione-*S*-transferase (GST).[2] Several members of the GST-isoenzyme family are involved in conjugation of drugs and thereby in drug resistance. Isoenzyme selective GST inhibitors may therefore be used to improve drug response and decrease resistance. GSH-conjugates and their cell-permeable esterified derivatives are effective competitive inhibitors of GST.[3,4] Unfortunately, the inhibitory action of GSH-conjugates is severely limited by their sensitivity towards peptidase-mediated breakdown. Although the γ-glutamyl-cysteine peptide bond renders it resistant to most α-peptidases, GSH is rapidly degraded by γ-glutamyl transpeptidase (γGT). Cleavage by this enzyme, which has particularly high activity in the kidney, prevents the use of GSH conjugates in vivo.

Being potential drug candidates, effort has been put into the design of new glutathione analogues as GST inhibitors. Several modifications have been made to the GSH peptide backbone, mostly by replacing the amino acid residues by other naturally occurring or synthetic analogues.[5–9] Recently, most of the known structural modifications of the glutathione molecule have been reviewed.[10] In recent years, our laboratory has been involved in the development of GSH analogues for the functional mapping and inhibition of GST isoenzymes. This resulted in the development of non-thiol containing *S*-alkyl-glutathione analogues, in which the cysteinyl-glycine part was replaced by D-aminoadipic acid. These compounds showed improved stability towards γ-GT and exhibited in vivo inhibition of some GST isoenzymes, although inhibition was limited to α and μ class GST isoenzymes.[11–15] In this study, new compounds

*Corresponding author. Tel.: +31-71-527-6227; fax; +31-71-527-6292; e-mail: d.burg@lacdr.leidenuniv.nl

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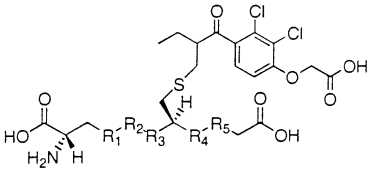
were designed that closely resemble GSH-conjugates, but in which native peptide bonds were replaced by peptidomimetics. These modified amides were expected to be less sensitive towards peptidase mediated breakdown. To increase inhibitory potency towards GST, the cysteine sulphydryl was conjugated to the diuretic drug ethacrynic acid (EA). The rationale behind this is that the GSH–ethacrynic acid conjugate is a potent inhibitor of most GST isoenzymes.[16,17] These compounds might therefore yield new lead compounds towards peptidase-stable GST inhibitors. Figure 1 shows the GSH-conjugate mimics synthesized for this purpose. Structure **I** depicts the native GSH conjugate, while compounds **II** and **III** indicate *N*-methylated tripeptides. In compound **IV**, an ethylene group replaces the cysteinyl-glycine amide, thereby introducing more flexibility to the peptide backbone. Amine **V** is a 'reduced' isostere; it lacks the carbonyl function and is therefore also expected to be protonated in physiological solutions. Synthesis of the peptide portion of the urethane **VI** has recently been described.[18] This GSH analogue was shown to be a competitive inhibitor of γGT, and its ethacrynic acid conjugate was therefore of much interest.

Results and Discussion

Chemistry

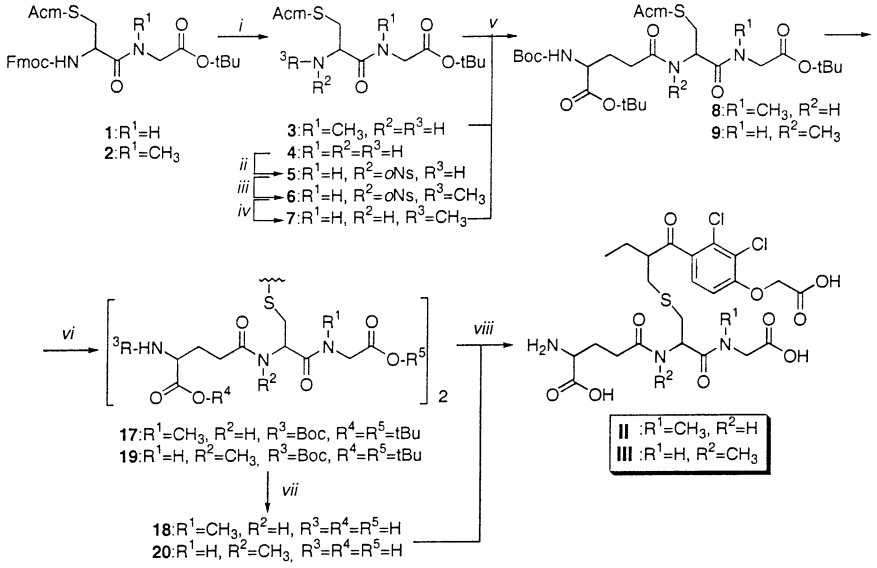
The reference compound **I**, was prepared by Michael addition of the α,β-unsaturated ketone moiety of ethacrynic acid (EA) to the cysteinyl-thiol of glutathione in a slightly basic water/ethanol mixture as has been previously described.[17] The product was homogeneic on HPLC and all spectroscopic data corresponded with literature values.

The synthetic route towards the *N*-methylated glutathione derivatives is shown in Scheme 1. Compound **II** was prepared by incorporating the *N*-methylamino acid sarcosine instead of the C-terminal glycine. The protected dipeptide, **2**, was obtained in high yield by condensing Fmoc-Cys(Acm)-OH with H-Src-OtBu, using DIC/HOBt as coupling agent. After removal of the N-terminal Fmoc (**3**), coupling to the unprotected side-chain carboxyl of Boc-Glu-OtBu was achieved via the same coupling method giving compound **8**. The Acm protecting group was removed with iodine in dry methanol.[18] The symmetrical disulfide, obtained by this oxidation was then treated with acid to remove Boc and *tert*-butyl protecting groups. In a one-pot reaction, the disulfide was reduced with tri-*n*-butylphosphine and coupled with EA, to give **II**. The resulting compound was epimeric at C_α of the ethacrynic acid moiety. During disulfide reduction and subsequent EA conjugation



	R1	R2	R3	R4	R5
I	CH_2	C=O	NH	C=O	NH
II	CH_2	C=O	NH	C=O	N-CH_3
III	CH_2	C=O	N-CH_3	C=O	NH
IV	CH_2	C=O	NH	CH_2	CH_2
V	CH_2	CH_2	NH	C=O	NH
VI	O	C=O	NH	C=O	NH

Figure 1. Structure of the glutathione–ethacrynic acid conjugate (**I**) and new peptidomimetic analogues. **I** = γ-Glu[Cys(EA)-Gly-OH]-OH (GS-EA), **II** = γ-Glu[Cys(EA)-Src-OH]-OH, **III** = γ-Glu[MeCys-Gly-OH]-OH, **IV** = γ-Glu[2-amino-1-mercapto(S-EA)-6-hexanoic acid]-OH, **V** = H-Glu[Ψ(CH2NH)-Cys(EA)-Gly-OH]-OH, **VI** = H-Glo[Cys(EA)-Gly-OH]-OH.

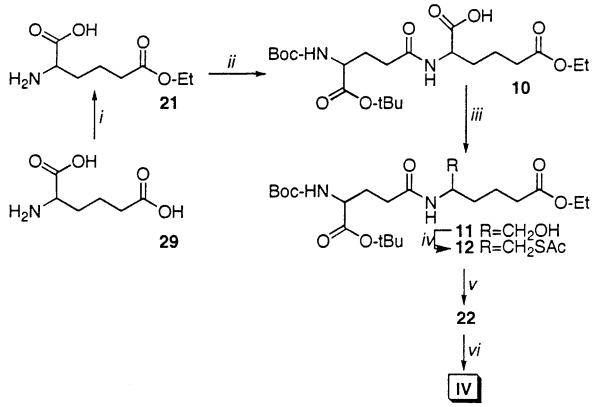


Scheme 1. Synthesis of *N*-methylated GSH-mimics. Conditions: (i). DBU, DCM; (ii). nitrobenzenesulfonylchloride, DiPEA, DCM; (iii). triphenylphosphine, DIAD, MeOH, THF, 0 °C; (iv). thiophenol, DiPEA, DMF; (v). **30**, HOBt, DIC, DCM; (vi). I_2, MeOH; (vii). TFA, 1% H_2O; (viii). Tri-*n*-butylphosphine, nPrOH/H_2O, ethacrynic acid.

a tri-*n*-butylphosphine–ethacrynic acid adduct was formed, as determined by LC–MS. This side-product was easily removed by reversed-phase HPLC. An identical adduct was also formed during the preparation of compounds **III**, **V** and **VI**.

N-Methylamide **III** was prepared by selective methylation of the cysteine amine. The liberated amino-group of cysteinyl-glycine was protected with *o*-nitrobenzenesulfonylchloride (**4**→**5**) to make the amine proton sufficiently acidic for Mitsunobu transformation.[19] Reaction of sulfonamide **5** with triphenylphosphine/DIAD and methanol gave **6** in high yield. The N-terminal *o*-nitrobenzylsulfonyl was cleaved with thiophenol and DiPEA in DMF (→**7**).[20] The *N*-methylated dipeptide could now be condensed with protected glutamic acid. Using DIC/HOBt as coupling agents, the *N*-methylated tripeptide **9** was obtained in moderate yields (60%). Because the coupling of *N*-methylated amino acids has often shown to be troublesome,[21] an attempt was made to improve the coupling efficiency. The use of DiPEA/PyBroP (an halogenated homologue of PyBOP[22]) as coupling reagent was not succesful, as this resulted in even lower yields (45%). Compound **III** was obtained after the same deprotection and conjugation steps as performed for compound **II**.

Synthesis of compound **IV** is shown in Scheme 2. Boc-Glu[-2-aminoadipic acid-OEt]-OtBu, **10,** was prepared by condensing D-aminoadipic acid ethyl ester with Boc-Glu-OtBu, as described by Ouwerkerk et al.[13] The adipic acid α-carboxyl was reacted with isobutylchloroformate to furnish a mixed anhydride, which was then reduced with $NaBH_4$ to give alcohol **11**.[23] Mitsunobu reaction with thioacetic acid transformed the dipeptide-alcohol into the thioacetate **12**. The thioacetate and the ethyl ester were cleaved simultaneously with aq NaOH in dioxane/MeOH. This was followed by conjugation of the free sulfhydryl with EA and subsequent acidolytic cleavage of Boc and tBu groups. Compound **IV** was obtained after purification by Sephadex LH20 gelfiltration.

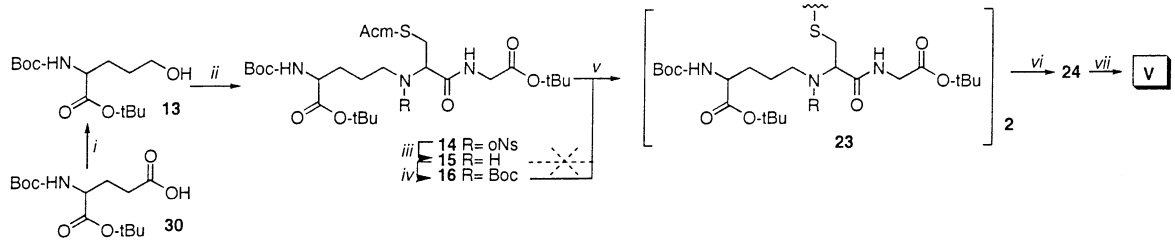


Scheme 2. Methylene replacement of the cysteinyl–glycine peptide bond. Conditions: (i). **29**, TMS-Cl, EtOH; (ii). BocGlu(OSu)OtBu, DiPEA, DMF; (iii). NMM, ethylchloroformate, $NaBH_4$, MeOH, THF; (iv). Triphenyl phosphine, DIAD, thioacetic acid, THF, 0 °C; (v). aq NaOH, dioxane, MeOH, ethacrynic acid.

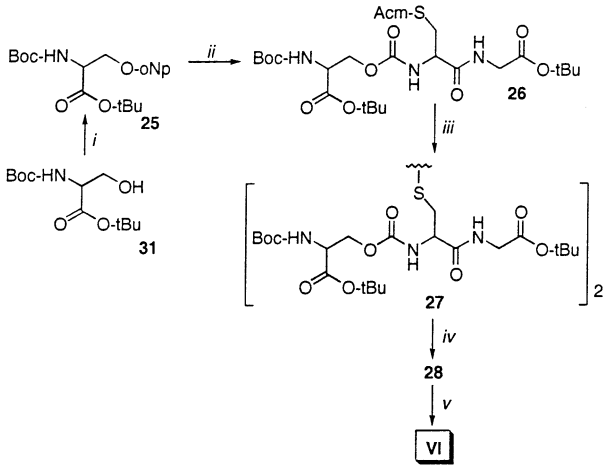
Reduced peptide isosteres are usually obtained after reductive amination of a peptide with a suitable peptide-aldehyde.[24] Although reduction of glutamic acid to the corresponding alcohol **13** proceeded in high yields (Scheme 3), subsequent oxidation to the aldehyde proved more difficult. Dess–Martin oxidation of the pure alcohol gave complex product-mixtures. No pure glutamic-aldehyde could be obtained via this method. In another attempt, the Weinreb amide of glutamic acid was prepared.[25] Reduction of this compound with $LiAlH_4$ also gave complex mixtures from which the product could not be isolated. A new route towards the reduced peptide isostere therefore had to be investigated.

This method is depicted in Scheme 3. In analogy with the methylated compound **III**, condensation of the glutamic alcohol **13** with the oNs-protected dipeptide **5** under Mitsunobu conditions gave the desired reduced peptide isostere **14**. After facile cleavage of the *o*-nitrobenzenesulfonyl protecting-group (→**15**), subsequent removal of Acm by iodine treatment led to complex mixtures of products on TLC. The free amine was therefore Boc-protected (→**16**) to prevent possible side-reactions and to facilitate purification. Indeed, the Acm group could now easily be removed to yield the symmetrical disulfide. After disulfide reduction, EA conjugation and TFA treatment compound **V** was isolated by Sephadex LH20 gelfiltration.

The reaction sequence towards urethane **VI** is depicted in Scheme 4. The peptide portion of this compound was prepared by a method adapted from Calcagni et al.[17] Boc-Ser(OH)-OtBu was acylated to yield its nitro phenylcarbonate (**25**), which was condensed with the amino group of H-Cys(Acm)-Gly-OtBu (**4**). The obtained urethane-based mimetic was then deprotected and con-



Scheme 3. Preparation of the reduced isostere. Conditions: (i). **30**, isobutylchloroformate, triethylamine, $NaBH_4$, THF/H_2O; (ii). triphenylphosphine, DIAD, **5**, THF; (iii). thiophenol, DiPEA, DMF; (iv). Boc_2O, DiPEA, MeCN; (v). I_2, MeOH; (vi). TFA, 1%H_2O; (vii). Tri-*n*-butylphosphine, $nPrOH/H_2O$, ethacrynic acid.



Scheme 4. Urethane isostere synthesis. Conditions: (i). **31**, bis(4-nitrophenyl)carbonate, DiPEA, DMF; (ii). **4**, dioxane, 80 °C; (iii). I_2, MeOH; (iv). TFA, 1%H_2O; (v). Tri-*n*-butylphosphine, nPrOH/H_2O, ethacrynic acid.

jugated to EA as was reported for the previously described compounds.

Biological evaluation

γ-Glutamyl transpeptidase mediated breakdown. Stability towards hydrolysis of the γ-glutamyl-cysteine amide by γGT was determined by incubating GS-EA analogues in the presence of the purified enzyme. Breakdown of the ethacrynic acid conjugates was determined by HPLC-analysis. All compounds were slightly unstable: After prolonged incubation (3 h) at 37 °C in Tris–HCl buffer, pH = 7.4, an unidentified peak was observed in the chromatograms, resulting from instability of the ethacrynic acid group (estimated breakdown $t_{1/2} > 10$ h).

This highly polar compound rapidly eluted from the column and did not interfere with peaks of the GSH analogues or their γGT-degraded products. As all GST inhibition studies are conducted using fresh solutions and lasted only 5 min, no loss of activity is expected as a result of this breakdown. All degradation data were corrected for the spontaneous breakdown. Figure 2 depicts the hydrolysis of the various GS-EA mimics by γGT.

As expected, the native glutathione–ethacrynic acid conjugate (**I**) was cleaved rapidly. The chosen conditions resulted in complete degradation of the GSH backbone within 20 min ($t_{1/2} = 8$ min). Compounds **II** and **IV**, each having an unmodified γ-glutamyl peptide bond, are also very sensitive towards γGT mediated cleavage. For these compounds, $t_{1/2}$ was approximately equal to the half-life of GS-EA.

The γ-glutamyl modified compounds (**III**, **V**, and **VI**) all showed greatly improved stability towards γGT. *N*-methylated compound **III** was hydrolyzed by γGT, albeit much slower than unmodified γ-glutamyl amides. After prolonged exposure to γGT, urethane **VI** also underwent minor hydrolysis. The reduced peptide isostere **V** was completely insensitive towards γ-glutamyl transpeptidase.

Inhibition of rat liver cytosolic GSTs

Rat liver cytosol (S100), containing cytosolic GSTs, was obtained after ultracentrifugation of a rat liver homogenate. GST inhibition was tested at six concentrations of the GS-EA mimics by a method adapted from Habig et al.,[26] modified for a 96-wells plate assay. Inhibition profiles of the various inhibitors are shown in Figure 3.

Rat liver cytosol contains mainly α (1-1, 2-2 and 1-2) and μ (3-3, 4-4, and 3-4) class GSTs. All peptidomimetics were able to inhibit GSH-conjugation by S100 GSTs, indicating that the compounds can be accommodated in the active sites of one or several isoenzymes. The unmodified glutathione conjugate (**I**) seemed to be the best GST inhibitor, inhibiting virtually all GSTs in the cytosol. Inhibition profiles indicate that most compounds reach a plateau at higher inhibitor concentration, which suggests that not all enzymes are inhibited equally. Disturbing the moieties involved in H-bonding has distinct consequences for enzyme–inhibitor interactions. The

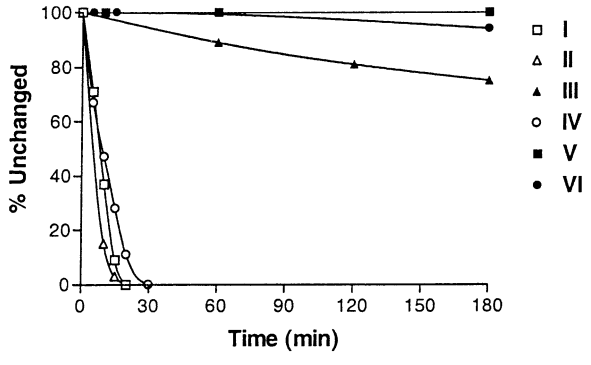


Figure 2. γ-Glutamyltranspeptidase mediated hydrolysis of GSH analogues. Peptide isosteres were incubated with 0.25 mg/ml bovine γ-glutamyltranspeptidase in 0.1 M Tris–HCl buffer pH = 7.4. At the appropriate time point a sample (100 μL) was taken, heat inactivated and stored on ice until HPLC analysis. Disappearance of the indicated compounds was determined by RP-HPLC. Open markers indicate γGT sensitive compounds, closed markers are γGT stabilized.

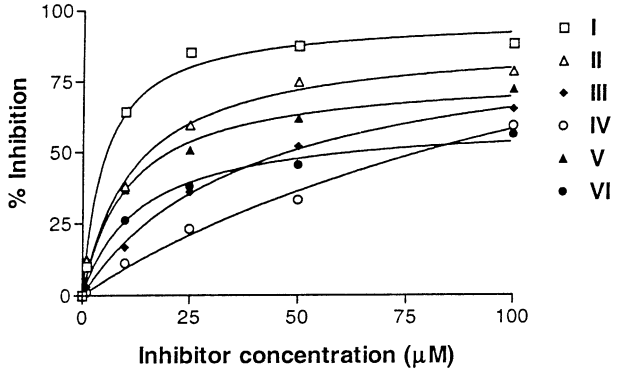


Figure 3. Inhibition of rat liver cytosolic GSTs (S100) by GS-EA mimics. Inhibition of GST mediated CDNB-conjugation was tested at pH = 6.5, in the presence of non-limiting concentrations of GSH (1 mM) and CDNB (1 mM). The shown data are averages of three separate determinations. For clarity, error-bars were not included. Standard deviations did not exceed 15% of the indicated averages. Open markers with dotted lines indicate γGT sensitive compounds (see Fig. 2), closed markers with closed lines are γGT stabilized structures.

GSH-binding site in GST is very tightly suited to accommodate GSH.[27] The binding capacity of the highly polar GSH-backbone is dictated by its electrostatic complementarity with the enzyme. Certain peptidomimetic changes to the GSH backbone can therefore result in drastically altered inhibition patterns.

Methylation of the cysteinyl-glycine amide (**II**) is very well accepted by cytosolic GSTs, indicated by a comparable inhibition pattern to compound **I**. The cys-gly amide nitrogen is presumably not directly involved in H-bonding. Furthermore, the increased steric bulk can be accommodated within the active site of the GST isoenzymes. Loss of water-bridged interactions may explain the slight decrease in inhibition. The importance of the γ-glutamyl-cysteine amide for enzyme–substrate interactions is clearly seen in compound **III**. When this peptide bond is methylated, a drastic decrease in inhibition was seen. This may be a result of the loss of a crucial H-bond, or may be a consequence of steric clashes with the enzyme. Compound **IV**, completely lacking the cysteinyl–glycine peptide bond is a poor inhibitor of cytosolic GSTs. The drop in inhibition is a result of omission of the carbonyl oxygen, which is an important H-bond acceptor.[28] The increased flexibility of the alkyl moiety is probably also an important factor, as enthropic effects may prevent this compound from obtaining its optimal bioactive conformation. Among the γGT stable inhibitors (**III**, **V** and **VI**), the reduced peptide **V** isostere seems to have the most favorable inhibition characteristics.

Conclusions

A series of pseudopeptide glutathione analogues was prepared by solution-phase peptide synthesis. Initial stability studies indicate that the γ-glutamyl modified compounds are stabilized against degradation by γ-glutamyltranspeptidase. Because this enzyme is mainly responsible for the degradation of GSH conjugates in the bloodstream, metabolic stabilization is a requisite for in vivo use of GSH conjugate analogues.

All ethacrynic acid conjugates inhibit rat liver cytosolic GSTs. Results indicate that alterations of the glutathione backbone are not equally well accepted by the different isoenzymes, and thus already provides isoenzyme selectivity. More elaborate inhibition studies on purified GST isoenzymes are needed to make firm conclusions regarding selectivity. The reduced peptide isostere may be considered as the most interesting lead for the development of in vivo active GST inhibitors, as is completely stable towards γ-GT and can be accommodated in the GST active site. In this study ethacrynic acid was used as model electrophile to obtain potent, but non-selective GSH conjugate analogues. Isoenzyme selectivity can be achieved by replacing the ethacrynic acid moiety by a more suitable hydrophobic substrate, thereby also avoiding the inherent instability of EA.

Apart from GST, several other enzymes such as glyoxalase I, DNA-dependent protein kinase, leukotriene-C_4-synthetase, and members of the MRP membrane-transporter family, are also inhibited by GSH-conjugates.[29–31] Because of the multitude of glutathione-dependent enzymes, γ-glutamyltranspeptidase stable GSH analogues might be interesting as research tools in various other fields of biological and biomedical research.

Experimental

Materials and methods

All solvents used were of analytical grade unless stated otherwise in the text. THF was freshly distilled prior to use. Other solvents were stored on molecular sieves. Protected amino acids and derivatives were purchased from Bachem AG (Bubendorf, Switzerland). Glutathione was purchased from Boehringer (Mannheim, Germany). Ethacrynic acid and bovine γ-glutamyltranspeptidase were obtained from Sigma-Aldrich (St. Louis, MO, USA).

For structure verification, NMR peaks were assigned by HH-, and CH-COSY experiments. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC-200, Bruker WM-300 or Bruker DMX-600 spectrometers, operating at 200/50.1, 300/75 and 600/150 MHz, respectively. Chemical shifts are given in ppm (δ) relative to the internal standard tetramethylsilane. Mass spectra were recorded with Finnigan MAT TSQ70 triple quadrupole, or with Perkin Elmer Sciex API 165 mass spectrometers. HPLC purification was performed on a Kratos spectroflow 400 system equipped with a Supelcosil SPLC-18-DB semi-preparative column. A linear gradient of 5-50% acetonitrile in H_2O/0.1%TFA was used to purify end products. For biological evaluation, all compounds were at least 90% pure, as determined by RP HPLC.

Chemical synthesis

Fmoc-Cys(Acm)-Src-OtBu (2). Fmoc-Cys(Acm)-OH (830 mg, 2 mmol) was added to a solution of HOBt (270 mg, 2 mmol) in 4 mL dry THF at 0 °C. A solution of sarcosine–OtBu·HCl (363 mg, 2 mmol) and *N*-methylmorpholine (NMM, 220 μL, 2 mmol) in 4 mL dichloromethane (DCM) was added in one portion. Diisopropylcarbodiimide (DIC, 310 μL, 2 mmol) was added dropwise to the reaction mixture. After 1 h at 0 °C, the suspension was stirred for 20 h at room temperature. DCM (50 mL) was added to the reaction mixture, which was then washed repeatedly with saturated $NaHCO_3$, 0.1 M HCl and water. Pure **2** was collected after silica gel column chromatography as white foam. Yield 1.05 g (97%). R_f 0.2 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.45 (9H, s, tBu), 1.98 (3H, CH_3Acm), 2.78–3.0 (2H, m, $C_\beta H_2$Cys), 3.16 (3H, s, CH_3Src), 3.75–4.1 (2H, m, CH_2Acm), 4.1–4.45 (3H, m, CH_2Src, C_αHCys), 4.2–4.45 (3H, m, CH–CH_2 Fmoc), 6.4 (1H, d, J = 9.1 Hz, NHCys), 7.24–7.75 (8H, m, aromatic), 7.4 (1H, m, NHAcm). ^{13}C NMR ($CDCl_3$): δ 23.06 (CH_3Acm), 27.67 (tBu), 33.28 (CH_2S), 36.31 (CH_3Acm), 41.37 (CH_2Acm), 50.44 (CH_2Src), 50.59

(C_αCys), 81.98 (C_qtBu), 119.7–128.07 (Fmoc), 140.96, 143.51 (Fmoc), 156.19 (CO Fmoc), 167.86 (CO ester), 170.89 (CO Cys), 171.59 (CO Acm).

Boc-Glu[Cys(Acm)-Src-OtBu]-OtBu (**8**). DBU (300 μL, 2 mmol) was added to a solution of the protected peptide **2** (1.05 g, 1.94 mmol) in DCM (10mL). After 15 min at room temperature, TLC analysis confirmed complete Fmoc removal. The solvent was evaporated under reduced pressure and the residue was co-evaporated with dry toluene. The crude H-Cys(Acm)-Src-OtBu (**3**) was used without further purification.

A solution of HOBt (270 mg, 2 mmol) and Boc-Glu-OtBu (**30,** 607 mg, 2 mmol) in 2 mL DCM was chilled and then added to an ice-cold solution of the amine **5** in DCM. DIC (310 μL, 2 mmol) was then added dropwise. The reaction mixture was stirred for two h at 0 °C and 16 h at 4 °C. The residue obtained after evaporation of the solvent was taken up in 100 mL EtOAc and washed with satd. aqueous $NaHCO_3$, 1 M $NaHSO_4$ and water. Silica gel column chromatography with 1% MeOH/DCM as eluent gave the pure tripeptide **8** as a foam, which solidified after further drying. Yield 995 mg (85%). R_f 0.45 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.4 (27H, Boc, tBu), 1.95 (3H, s, CH_3Acm), 1.65–2.3 (2H, m, $C_\beta H_2$Glu), 1.97 (3H, s, CH_3Acm), 2.29 (2H, m, $C_\gamma H_2$Glu), 2.6–3.05 (2H, m, $C_\beta H_2$Cys), 3.12 (3H, s, CH_3Src), 3.68–4.1 (2H, m, CH_2Acm), 4.2 (2H, m, CH_2Src), 4.35 (1H, m, C_αHGlu), 5.08 (1H, m, C_αHCys), 5.38 (1H, d, NHBoc), 7.05 (1H, d, NHCys), 7.45 (1H, d, NHAcm). ^{13}C NMR ($CDCl_3$/MeOD): δ 22.57 (CH_3Acm), 27.6, 27.97 (Boc, tBu), 31.7 (C_βGlu), 33.36 (C_βCys), 36.3 (CH_3Src), 41.49 (CH_2Acm), 48.7 (C_αCys), 50.4 (CH_2Src), 52.04 (C_γGlu) 53.38 (C_αGlu), 81.6–82.6 (C_q, Boc, tBu), 155.68 (CO, Boc), 167.8 (CO, Src-tBu ester), 170.8–172.24 (CO, Acm, Cys, Glu, Glu-tBu ester). Mass spectrometry (ES-MS): *m/e:* 60, $[M+H]^+$; 627, $[M+Na]^+$.

{Boc-Glu[Cys-Src-OtBu]-OtBu}$_2$ (**17**). Peptide **8** (603 mg, 1 mmol) was dissolved in 10 mL MeOH. A solution of I_2 (508 mg, 2 mmol) in 5 mL MeOH was added dropwise over 30 min. After 10 min TLC analysis indicated complete disulfide formation. The solution was decolorized by addition of 1 N sodium thiosulfate solution. EtOAc (100 mL) was added and the organic phase was washed with thiosulfate solution and water. After evaporation of the solvent, product **17** was purified by gelfiltration chromatography on a Sephadex LH20 column, using DCM/MeOH (1:1) as solvent. Yield 458 mg (86%). R_f 0.6 (DCM/MeOH, 9:1). ^{13}C NMR ($CDCl_3$): δ 27.22, 27.55 (Boc, tBu), 31.46 (C_βCys), 35.92 (CH_3Src), 39.37 (C_βGlu), 48.05 (C_αCys), 50.17 (CH_2Src), 51.62 (C_γGlu), 53.45 (C_αGlu), 79.13-82.26 (C_q, Boc, tBu), 155.73 (CO, Boc), 167.5 (CO, Src-OtBu ester), 170.6–172.14 (CO, Cys, Glu, Glu-OtBu ester).

{H-Glu[Cys-Src-OH]-OH}$_2$ (**18**). Trifluoroacetic acid (5 mL) and water (100 μL) was added to **17** (458 mg, 0.43 mmol). After 4 h at room temperature, the deprotected disulfide **18** was precipitated by addition of ice-cold diethylether. The product was repeatedly coevaporated with ether and used without further purification. Yield 276 mg (100%). ^{1}H NMR (MeOD): δ 2.2 (4H, m, $C_\beta H_2$Glu), 2.55 (4H, m, $C_\gamma H_2$Glu), 2.85 and 3.2 (4H, m, $C_\beta H_2$Cys), 2.9 (2H, s, C_αHGlu) 3.25 (3H, s, CH_3Src), 4.05 (4H, m, CH_2Gly), 4.1–4.6 (2H, m, C_αHCys), 8.55 (2H, d, NHamide). ^{13}C NMR (MeOD): δ 32.1 (C_βCys), 36.9 (CH_3Src), 40.6 (C_βGlu), 50.0 (C_αCys), 50.5 (CH_2Src), 51.3 (C_γGlu), 53.4 (C_αGlu), 171.5–173.8 (4×C=O).

H-Glu[Cys(EA)-Src-OH]-OH, II. Compound **18** was dissolved in 6 mL argon flushed nPrOH/H_2O (2:1). The pH was adjusted to 8.5 with 25% aq NH_3 solution. Tri-*n*-butylphosphine (106 mg, 0.43 mmol) was added, after which the solution was stirred under argon for 60 min. Ethacrynic acid (303 mg, 1 mmol), dissolved in 2 mL EtOH was then added. The pH was adjusted to 8 by addition of satd. $NaHCO_3$ solution. After at least 4 h, the solvent was evaporated. The residue was further purified by LH20 gelfiltration using MeOH/H_2O (7:3) as eluent. LC–MS confirmed the presence and identity of the desired compound **II**. Residual traces of tri-*n*-butylphosphine–EA complex were removed by RP-HPLC. R_f 0.3 (nBuOH/H_2O/AcOH, 15:3:2). ^{1}H NMR (D_2O): δ 0.85 (3H, m, CH_3EA), 1.55 and 1.71 (2H, m, CH_2EA), 2.11 (2H, m, $C_\beta H_2$Glu), 2.45 (2H, m, $C_\gamma H_2$Glu), 2.67–2.8 and 2.85–3.0 (2H, m, $C_\beta H_2$Cys), 2.9 (2H, d, $J=6.3$ Hz, CH_2EA), 3.1 (3H, s, CH_3Src), 3.53 (1H, m, CH-EA), 3.74 (1H, m, C_αHGlu), 3.8–4.06 (2H, m, CH_2Src), 4.63 (2H, s, CH_2EA), 4.74–4.95 (1H, C_αHCys), 6.95 and 7.60 (2×1H, m, aromatic). ^{13}C NMR: 11.45 (CH_3EA), 25.4 (4×CH_2, C_βGlu, C_βCys, 2×CH_2EA), 37.4 (CH_3Src), 50.3 (CH EA), 52.5 (C_αCys), 53.1 (CH_2Src), 54.5 (C_γGlu), 56.5 (C_αGlu), 68.6 (OCH_2EA), 111.9 and 129.7 (CH, aromatic), 123.9 and 131.95 (C–Cl, aromatic), 158.1–175.65 (5×CO). Mass spectrometry (ES–MS): *m/e* 624, $[M+H]^+$.

Fmoc-Cys(Acm)-Gly-OtBu (**1**). To a solution of Fmoc-Cys(Acm)-OH (829 mg, 2 mmol) in 4 mL dry THF at 0 °C, HOBt (270 mg, l2 mmol) was added. Then an ice-cold solution of H-Gly-OtBu.HCl (335 mg, 2 mmol) and NMM (220 mg, 2 mmol) in 4 mL of dry DCM was added. Subsequently, DIC (310 mg, 2 mmol) was introduced dropwise. After 1 h at 0 °C and 16 h at 4 °C, the solution was evaporated under vacuum. The residue was dissolved in 50 mL EtOAc and washed with satd. aq $NaHCO_3$, 0.5 M HCl and water. After drying and evaporation of the organic phase, the residue was chromatographed on silica gel to give the protected dipeptide **1** as a foam. Yield 1.03 g (98%), R_f 0.42 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCL_3$): δ 1.45 (9H, s, tBu), 1.92 (3H, s, CH_3 Acm), 2.97 (2H, m, H_β Cys), 3.96 (2H, m, Acm), 4.2–4.5 (3H, m, CH–CH_2 Fmoc), 4.25–4.65 (3H, H_α Cys, CH_2 Gly), 6.47 (1H, d, $J=8.4$ Hz), 7.3 (2H, 2×NH), 7.24–7.74 (8H, m, aromatic). ^{13}C NMR ($CDCl_3$): δ 23.15 (CH_3, Acm), 27.7 (3×CH_3, tBu), 33.4 (C_β, Cys), 40.6 (CH_2, Acm), 41.9 (CH_2, Fmoc), 81.8 (C_q, tBu), 119.7–127.5 (aromatic, Fmoc), 141.0 and 143.6 (4C, Fmoc), 156.6 (CO, Fmoc), 168.6 (CO, Cys), 171.6 (2× CO, Acm, tBu ester).

H-Cys(Acm)-Gly-OtBu (**4**). Compound **1** (791 mg, 1.5 mmol) was dissolved in 5 mL dry DCM. DBU (224 mg,

1.5 mmol) was added at room temperature. After 15 min at room temperature, the solution was evaporated under reduced pressure. The crude product was used without further purification. R_f 0.41 (DCM/MeOH, 9:1).

oNs-Cys(Acm)-Gly-OtBu (**5**). The crude Fmoc deprotected dipeptide **4** (456 mg, 1.5 mmol) was dissolved in 10 mL dry DCM. The solution was cooled on an ice/water bath, after which DiPEA (311 mg, 1.8 mmol) and 2-nitrobenzenesulfonylchloride (399 mg, 1.8 mmol) were added. After 16 h at ambient temperature, the solvent was evaporated and product **5** was purified by silica gel column chromatography. Yield 525 mg (74% over two steps). R_f 0.38 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.45 (9H, s, tBu), 2.04 (3H, s, CH_3Acm), 2.86 (2H, m, $C_\beta H_2$Cys), 3.75 (2H, m, CH_2Acm), 4.12 (1H, C_αHCys), 4.5 (2H, m, CH_2Gly), 6.95 (1H, m, NHGly), 7.45 (1H, m, NHAcm), 7.6 (1H, m, NH-oNS), 7.72–8.18 (4H, m, aromatic).

oNs-MeCys(Acm)-Gly-OtBu (**6**). DIAD (223 μL, 1.15 mmol) was added to a stirred solution of triphenylphosphine (302 mg, 1.15 mmol) in dry 3 mL THF at 0 °C. After 30 min, a white precipitate had formed, indicating the formation of the phosphonium intermediate. Peptide **5** (283 mg, 0.6 mmol) and 1.2 mmol dry MeOH (50 μL), dissolved in 1 mL dry THF, were added dropwise to the ice-cold reaction mixture. The solution was stirred for 2 h at 0 °C, and for 24 h at room temperature. The solvent was evaporated, and pure **6** was obtained after silica gel column chromatography. Yield > 95%. R_f 0.42 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.47 (9H, s, tBu), 2.04 (3H, s, CH_3Acm), 2.68–3.27 (2H, m, $C_\beta H_2$Cys), 3.03 (3H, s, CH_3N), 3.69–4.04 (2H, m, CH_2Acm), 4.17 (1H, m, C_αHCys), 4.5 (2H, m, CH_2Gly), 6.79 (1H, m, NHGly), 7.25 (1H, m, NHAcm), 7.4–8.15 (4H, m, aromatic).

H-MeCys(Acm)-Gly-OtBu (**7**). Thiophenol (256 μL, 2.5 mmol) and DiPEA (345 μL, 2 mmol) were added to a solution of **6** (243 mg, 0.5 mmol) in 5 mL DMF. After 16 h at room temperature, TLC analysis indicated complete oNs-cleavage. Toluene (5 mL) was added to the reaction mixture. The DMF/toluene mixture was added on a silica gel column, which was equilibrated with pure toluene. The column was flushed until the bright yellow apolar o-Ns cleavage products and excess thiophenol had eluted. Elution with a gradient of EtOAc in toluene gave the methylated dipeptide **7** as an oil. Yield 156 mg (98%). R_f 0.14 (DCM/MeOH, 95:5). NMR analysis confirmed complete removal of oNs group.

Boc-Glu[MeCys(Acm)-GlyOtBu]-OtBu (**9**). Boc-Glu(OH)-OtBu(**30**, 152 mg, 0.5 mmol) and **7** (156 mg, 0.5 mmol) were added to a stirred solution of HOBt (68 mg, 0.5 mmol) in 5 mL dry DCM at 0 °C. DIC (77 μL, 0.5 mmol) was then slowly added to the solution. After 24 h at room temperature, the solvent was evaporated and the residue was dissolved in 100 mL EtOAc. The organic layer was then washed repeatedly with satd.aq $NaHCO_3$ solution, 0.1 M HCl solution and water. Removal of the solvent and purification over a silica gel column gave the pure *N*-methylated tripeptide **9** as an oil. Yield 181mg (60%). R_f 0.4 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.45 (27H, s, Boc, tBu), 1.89 (2H, m, $C_\beta H_2$Glu), 2.05 (3H, s, CH_3Acm), 2.45 (2H, m, $C_\gamma H_2$Glu), 2.97 (3H, s, N–CH_3), 2.22–3.27 (2H, m, $C_\beta H_2$Cys), 3.85 (2H, d, J = 5.8 Hz, CH_2Gly), 4.24 (1H, m, C_αHGlu), 4.31–4.65 (2H, m, CH_2Acm), 5.32 (1H, m, C_αHCys), 5.4 (1H, d, J = 8.0 Hz, NHBoc), 7.11 (1H, m, NHGly), 7.33 (1H, m, NHAcm).

{Boc-Glu[MeCys-GlyOtBu]-OtBu}$_2$ (**19**). Product **19** was prepared as described for compound **17**. Yield 143 mg (89%). R_f 0.45 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.45 (54H, s, Boc, tBu), 1.9 (4H, m, $C_\beta H_2$Glu), 2.45 (4H, m, $C_\gamma H_2$Glu), 2.19–3.36 (4H, m, C_βCys), 3.02 (6H, s, N–CH_3), 3.88 (4H, d, J = 5.8 Hz, CH_2Gly), 4.19 (2H, m, C_αHGlu), 5.27 (2H, d, J = 8.05 Hz, NHBoc), 5.3 (2H, m, C_αHCys), 7.51 (2H, m, NHGly).

{H-Glu[MeCys-Gly-OH]-OH}$_2$ (**20**). Acid-labile protecting groups were removed as described for compound **18**. Product **20** was obtained after triturating with diethyl ether and used without further purification.

H-Glu[MeCys(EA)-Gly-OH]-OH, III. Disulfide reduction and ethacrynic acid conjugation was performed essentially the same as for compound (**I**). Compound **II** was purified after LH20 gelfiltration (70% MeOH/water) and RP-HPLC. ^{1}H NMR (D_2O): δ 0.85 (3H, m, CH_3EA), 1.5–1.7 (2H, m, CH_2EA), 2.2 (2H, m, $C_\beta H_2$Glu), 2.45 (2H, m, $C_\gamma H_2$Glu), 2.2–3.2 (2H, m, $C_\beta H_2$Cys), 2.9 (2H, d, J = 6.3 Hz, CH_2EA), 2.95 (3H, s, N–CH_3), 3.5 (1H, m, CH–EA), 3.7 (1H, m, C_αHGlu), 3.9 (2H, d, CH_2Gly), 4.63 (2H, s, CH_2EA), 5.3 (1H, C_αHCys), 6.95 and 7.60 (2×1H, m, aromatic). Mass-spectrometry (ES-MS): m/e 624, $[M+H]^+$.

D-2-Aminoadipicacid-OEt (**21**). D-2-aminoadipic acid **29** (1.61g, 10mmol) was suspended in 30 mL dry ethanol in an N_2 atmosphere. TMS-Cl (2.78 mL, 22 mmol) was added dropwise to the suspension. The resulting clear solution was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure and the crude ester was redissolved in 60 mL EtOH. Pyridine (4.5 mL) was added, after which a white precipitate formed immediately. After 6 h at 4 °C, the crystals were collected and washed with ether. Recrystallization from water containing a trace of pyridine yielded pure monoester **21** as a white solid. Yield 1.569 g (83%). ^{1}H NMR (D_2O): δ 1.24 (3H, t, J = 7.2 Hz, CH_3ethyl), 1.72 (2H, m, $C_\gamma H_2$), 1.9 (2H, m, $C_\beta H_2$), 2.45 (2H, m, $C_\delta H_2$), 3.92 (1H, t, J = 5.9 Hz, C_αH), 4.15 (2H, q, CH_2ethyl).

Boc-Glu[Aminoadipicacid-OEt]-OtBu (**10**). Compound **21** (945mg, 5mmol) was suspended in 50 mL DMF, containing DiPEA (950 μL, 5.5 mmol) and Boc-Glu(OSu)-OtBu (5 mmol). The mixture was stirred overnight, after which the clear solution was concentrated under reduced pressure and acidified to pH = 3 with 1M HCl. EtOAc (200 mL) was added and the organic phase was washed with water. The organic layer was dried over $MgSO_4$ and evaporated. Pure dipeptide **10** was obtained as colorless oil after silica gel

column chromatography. Yield 85%. R_f 0.63 (DCM/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ 1.25 (3H, t, $J=7.2$ Hz, CH_3Ethyl), 1.4 (18H, s, Boc, tBu), 1.7 (4H, m, $C_\beta H_2$Aad, $C_\gamma H_2$Aad), 1.9 (2H, m, $C_\beta H_2$Glu), 2.35 (4H, m $C_\gamma H_2$Glu, $C_\delta H_2$Aad), 3.95 (1H, m, C_αHAad), 4.1 (2H, q, CH_2ethyl), 5.55 (1H, d, NHBoc), 7.25 (1H, d, NHamide).

Boc-Glu[2-amino-1-hydroxy-6-hexanoicacid-OEt]-OtBu (11). Compound **10** (1 mmol) was dissolved in 5 mL THF at −10 °C. NMM (110 μL, 1 mmol) and ethylchloroformate (96 μL, 1 mmol) were added and the resulting suspension was stirred for 15 min at 0 °C. $NaBH_4$ (121 mg, 3.2 mmol) was added in one portion. Methanol was added dropwise, carefully controlling the rate of reduction. After addition of 10 mL methanol, the mixture was allowed to stir for 30 min at room temperature. The pH of the solution was acidified (pH 5) by addition of 1M aq H_2SO_4. EtOAc (50 mL) was added and the resulting solution was washed with water. The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica gel column chromatography, using EtOAc as eluent. The pure dipeptide alcohol **11** was obtained as colorless oil. Yield 84%. R_f 0.2 (EtOAc/hexanes/AcOH, 10:10:1). ^{1}H NMR ($CDCl_3$): δ 1.25 (3H, t, $J=7.2$ Hz, CH_3ethyl), 1.44–1.47 (18H, 2×s, Boc, tBu), 1.52–1.77 (6H, m, $C_\beta H_2$, $C_\gamma H_2$ Hex + $C_\beta H_2$Glu), 2.07–2.36 (4H, $C_\gamma H_2$ Glu + $C_\delta H_2$ Hex), 3.53–3.78 (2H, CH_2O), 3.9 (1H, m, C_αH Hex), 4.08–4.17 (3H, m, CH_2ethyl + C_αH Glu), 5.4 (1H, d, $J=8.2$ Hz, NH Boc), 6.5 (1H, d, $J=8.2$ Hz, NH amide). ^{13}C NMR ($CDCl_3$): δ 13.91 (CH_3Et), 21.1 (C_γHex), 28.0 (Boc, tBu), 28.67 (C_βGlu), 30.13 (C_γGlu), 32.28 (C_βHex), 33.59 (C_δHex), 50.99 (C_αHex), 53.21 (C_αGlu), 60.03 (CH_2ethyl), 64.21 (CH_2–OH), 79.62, 81.77 (C_q, Boc, tBu), 155.80 (CO Boc), 171.32, 172.32, 173.29 (CO).

Boc-Glu[2-amino-1-mercaptoacetyl-6-hexanoicacid-OEt]-OtBu (12). DIAD (291 μL, 1.5 mmol) was added to a stirred solution of triphenylphosphine (394 mg, 1.5 mmol) in 3.5 mL dry THF at 0 °C. A white precipitate formed immediately. After 30 min at 0 °C, a solution of peptide alcohol **11** (0.75 mmol) and thioacetic acid (107 μL, 1.5 mmol) in 1.5 mL THF was added dropwise to the suspension. After 1 h at 0 °C, the solution was stirred for 16 h at room temperature. The solvent was evaporated under reduced pressure. The residue was subjected to silica gel column chromatography, using EtOAc/hexanes (3:7) as eluent. Product **12** was collected as a colorless oil. Yield 89%. R_f 0.52 (EtOAc/hexanes, 1:1). ^{1}H NMR ($CDCl_3$): δ 1.25 (3H, t, $J=7.2$ Hz, CH_3ethyl), 1.45 (18H, 2×s, Boc, tBu), 1.5–1.95 (5H, m, $C_\beta H_2$Hex, $C_\gamma H_2$Hex, $C_\beta H_2$Glu), 2.05–2.37 (7H, m, CH_3SAc, $C_\delta H_2$Hex, $C_\gamma H_2$Glu), 3.05 (2H, m, CH_2–S), 4.1 (3H, m, CH_2ethyl, C_αHHex), 5.29 (1H, d, $J=7.2$ Hz, NHBoc), 6.45 (1H, d, $J=7.2$ Hz, NHamide). ^{13}C NMR: δ 13.9 (CH_3 ethyl ester), 20.97 (C_γHex), 27.6, 28.0 (Boc, tBu), 30.15 (CH_3 SAc), 32.25–33.37 ($C_\beta C_\gamma$Glu, CH_2-S, $C_\beta C_\delta$Hex), 48.56 (C_αHex), 53.57 (C_αGlu), 59.9 (CH_2ethyl ester), 79.13 (C_qBoc), 81.34 (C_q tBu), 155.61 (CO, Boc), 171.23, 171.8 (2×CO, ester), 172.89 (CO Glu), 195.15 (CO, SAc). Mass spectrometry *m/e* (EI-MS): 475, 443, 417, 389, 361, 329, 317, 300, 273 and smaller fragments. Fragmentation is in accordance with the expected structure.

Boc-Glu[2-amino-1-mercapto(S-EA)-6-hexanoicacid]-OtBu (22). The thioacetate moiety and the ethyl ester of compound **12** were simultaneously cleaved using 5 equivalents of Tesser's base (Dioxane/MeOH/2 M aq NaOH, 15:4:1) in an argon atmosphere. After 3 h at room temperature, ethacrynic acid (303 mg, 1 mmol) in 5 mL ethanol was added to the mixture. The mixture was allowed to stir for at least 4 h. The pH was then brought to 3 with 1 M HCl solution. Solvents were evaporated under reduced pressure. The residue was dissolved in ethyl acetate and washed several times with 0.1M HCl solution. The ethacrynic acid conjugate **22** was collected by evaporation of the organic layer and was used without further purification. R_f 0.45 (DCM/MeOH/AcOH, 95:5:1).

γ-Glu[2-amino-1-mercapto(S-EA)-6-hexanoicacid-OEt]-OH, IV. Trifluoroacetic acid/1%H_2O (10 mL) was added to **22**. After 3 h at room temperature, ice-cold diethylether was added. The precipitated product **IV** was washed with diethylether and further purified by Sephadex LH20 gelfiltration, using MeOH/water (7:3) as eluent. R_f 0.30 (nBuOH/H_2O/AcOH, 15:3:2). ^{1}H NMR (D_2O): δ 0.86 (3H, t, CH_3EA), 1.35–1.9 (6H, m, CH_2EA, $C_\gamma H_2$Glu, $C_\beta H_2$Hex), 2.1–2.35 (4H, m, $C_\beta H_2$Glu, $C_\delta H_2$Hex), 2.37–2.4 (2H, $C_\gamma H_2$Glu), 2.52–2.95 (4H, m, CH_2EA, CH_2–S), 3.56 (1H, CHEA), 3.74–3.86 (2H, C_αHGlu, C_αHHex), 4.65 (2H, s, OCH_2 EA), 6.95 (1H, d, Arom), 7.60 (1H, d, Arom.). Mass spectrometry (ES–MS) *m/e:* 595, $[M+H]^+$.

Boc-Glutaminol-OtBu (13). Boc-Glu-OtBu (**30,** 304 mg, 1 mmol) was dissolved in 3 mL freshly distilled THF and cooled to −40 °C under a stream of N_2 gas. Triethylamine (167 μL, 1.2 mmol) was added to the cold solution, followed by dropwise addition of isobutylchloroformate (157 μL, 1.2 mmol) in 1 mL THF. The resulting suspension was stirred at −20 °C for 45 min. The suspension was then filtered into a rigorously stirred solution of $NaBH_4$ in 2mL THF/H_2O (8:1) at 0 °C. The reduction was then allowed to stir for 3 h at room temperature. The solvent was then removed under reduced pressure and compound **13** was purified by silica gel column chromatography, using EtOAc/hexanes (1:1) as eluent. Yield 257mg (89%). R_f 0.4 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.45 (18H, 2×s, Boc, tBu), 1.54–1.95 (4H, m, $C_\beta H_2$, $C_\gamma H_2$), 3.65 (2H, t, $J=5.9$ Hz, $C_\delta H_2$), 4.16 (1H, m, C_αH), 5.34 (1H, d, $J=8.4$ Hz, NH).

Boc-Glu[Ψ (CH_2N-oNS)-Cys(Acm)-Gly-OtBu]-OtBu (14). To a stirred solution of triphenylphosphine (394 mg, 1.5 mmol) in 15 mL freshly distilled THF, DIAD (291 μL, 1.5 mmol) was added at −50 °C in an argon atmosphere. After 15 min, a white suspension had formed, to which a solution of alcohol **13** (257 mg, 0.89 mmol) and oNs-protected dipeptide **5** (425 mg, 0.9 mmol) in 7 mL THF was added dropwise. The temperature was carefully maintained at −50 °C during the peptide/alcohol addition. Afterwards, the reaction

mixture was slowly allowed to reach room temperature and was stirred overnight. The solvent was removed and product **14** was purified over a silica gel column. The product was collected as an oil. Yield 630 mg (96%). R_f 0.65 (DCM/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ 1.45 (27H, s, Boc, tBu), 1.5–1.85 (4H, m, $C_\beta H_2$Glu, $C_\gamma H_2$Glu), 1.97 (3H, s, CH_3Acm), 2.69–3.25 (2H, m, $C_\beta H_2$Cys), 3.63–3.97 (2H, m, CH_2Acm), 4.03 (2H, m, CH_2Gly), 4.45 (1H, m, C_αHGlu), 4.76 (1H, m, C_αH-Cys), 5.2 (1H, d, $J=7.2$ HZ), 7.39–8.15 (5H, NHGly, CH–aromatic). ^{13}C NMR ($CDCl_3$): δ 22.82 (CH_3Acm), 26.3 (C_γGlu), 27.82, 28.15 (Boc, tBu), 29.82 (C_βGlu), 31.3 (C_βCys), 40.56 (CH_2Acm), 42.0 (CH_2Gly), 45.59 (C_δGlu), 53.32 (C_αGlu), 59.18 (C_αCys), 79.37, 81.77 (C_q Boc, tBu), 124.0–133.56 (4C, aromatic), 147.85 (C-NO_2), 155.34 (CO Boc), 168.2-171.59 (4×CO).

Boc-Glu[Ψ (CH_2NH)-Cys(Acm)-Gly-OtBu]-OtBu (15). Compound **14** (584 mg, 0.8 mmol) was dissolved in 15 mL dry DMF in an N_2 atmosphere. Thiophenol (409 μL, 4 mmol) and DiPEA (552 μL, 3.2 mmol) were added, after which the mixture was stirred overnight. Toluene was added to the reaction mixture and applied to a silica gel column. Flushing with toluene was continued until excess thiophenol and brightly colored deprotection by-products had eluted. The eluent was then replaced by EtOAc/hexanes, after which product **15** could be isolated as an oil. Yield 436 mg (100%). R_f 0.26 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCL_3$): δ 1.41 (27H, ss, Boc, tBu), 1.54 (2H, m, $C_\gamma H_2$Glu), 1.74 (2H, m, $C_\beta H_2$Glu), 1.98 (3H, s, CH_3Acm), 2.52–3.05 (2H, $C_\beta H_2$Cys), 2.85 (2H, s, $C_\beta H_2$Cys), 2.93 (2H, m, $C_\delta H_2$Glu), 3.6 and 3.69 (1H, m, C_αHCys), 3.89 (2H, d, $J=5.9$ Hz, CH_2Gly), 4.18 (1H, m, C_αHGlu), 4.3 (2H, m, CH_2Acm), 5.2 (1H, d, $J=8.4$ Hz, NHBoc), 7.19 (1H, m, NHAcm), 7.92 (1H, m, NHGly).

Boc-Glu[Ψ (CH_2N-Boc)-Cys(Acm)-Gly-OtBu]-OtBu (16). Reduced peptide isostere 15 (436 mg, 0.8 mmol) was dissolved in 20 mL dry acetonitrile. DiPEA (0.155 μL, 0.9 mmol) and BOC_2O (350 mg, 1.6 mmol) were added. The mixture was stirred at 60 °C for 24–48 h, until no starting material could be detected by TLC. EtOAc (100 mL) was added and the organic phase was washed with satd aq $NaHCO_3$, 0.1 M HCl and water. The organic solvent was evaporated under reduced pressure. Compound **16** was obtained as an oil after silica gel column chromatography, using EtOAc/hexanes as eluent. Yield 516 mg (100%). R_f 0.3 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.45 (27H, ss, Boc, tBu), 1.5–1.8 (4H, m, $C_\beta H_2$Glu, $C_\alpha H_2$Glu), 2.01 (3H, s, CH_3Acm), 2.85 and 3.2 (2H, m, $C_\beta H_2$Cys), 3.17 (2H, m, $C_\delta H_2$Glu), 3.9 (2H, m, CH_2Gly), 4.23 (1H, m, C_αHGlu), 4.54 (2H, m, CH_2Acm), 4.67 (1H, m, C_αHCys), 5.18 (1H, d, $J=8.0$ Hz) 6.9 (2H, m, NHAcm, NHGly). ^{13}C NMR ($CDCl_3$/MeOD): δ 21.94 (CH_3Acm), 25.03 (C_γGlu), 27.27–27.61 (Boc, tBu), 28.94 (C_βGlu), 29.97 (C_βCys), 40.2 (CH_2Acm), 41.32 (CH_2Gly), 44.32 (C_δGlu), 53.66 (C_αGlu), 57.84 (C_αCys), 78.0–81.2 (4× C_q, Boc, tBu), 155.55, 156.03 (2×COBoc), 168.2–171.53 (4×CO).

{Boc-Glu[Ψ (CH_2N-Boc)-Cys-Gly-OtBu]-OtBu}$_2$ (23). Acm-deprotection was performed as described for compound **17**. Yield 93%. R_f 0.45 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCL_3$): δ 1.45 (72H, s, Boc, tBu), 1.5–1.82 (8H, m, $C_\beta H_2$Glu, $C_\gamma H_2$Glu), 2.8–3.6 (8H, m, $C_\delta H_2$Glu, $C_\beta H_2$Cys), 3.9 (4H, d, CH_2Gly), 4.12 (2H, m, C_αHGlu), 4.9 (2H, m, C_αHCys), 5.15 (2H, d, NHBoc), 7.45 (2H, m, NHGly).

Boc-Glu[Ψ (CH_2N-Boc)-Cys(EA)-Gly-OtBu]-OtBu (24). The protected disulfide 23 (0.37 mmol) was dissolved in 5 mL nPrOH/H_2O (4:1). The pH was adjusted to 8.5 with 25% aq NH_4OH solution and the mixture was flushed with argon. After addition of tri-*n*-butylphosphine (92 μL, 0.37 mmol), the resulting mixture was stirred for 1 h at room temperature. Ethacrynic acid (303 mg, 1 mmol), dissolved in 1 mL EtOH was added. After at least 4 h at rt, the solvent was evaporated. The residue was purified by silica gel column chromatography, using DCM/MeOH (95:5) as eluent. The protected EA conjugate was collected as an oil. Yield 389 mg (65%). R_f 0.35 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 0.9 (3H, t, CH_3EA), 1.45 (36H, s, Boc, tBu), 1.5–1.89 (6H, m, $C_\beta H_2$Glu, $C_\gamma H_2$Glu, CH_2EA), 2.15–2.7 (2H, m, $C_\beta H_2$Cys), 2.97–3.4 (2H, m, $C_\delta H_2$Glu), 3.57 (1H, m, CHEA), 3.9 (2H, d, CH_2Gly), 4.2 (1H, m, C_αHGlu), 4.78 (2H, s, CH_2EA), 5.15 (1H, d, NHBoc), 6.78, 7.15 (2×1H, dd, EA aromatic).

H-Glu[Ψ (CH_2NH)-Cys(EA)-Gly-OtBu]-OH, V. Deprotection of **24** was performed by addition of 5 mL TFA/H_2O (99:1). After 4 h at rt, the product was precipitated by addition of ice-cold diethylether. Further purification of the precipitate by Sephadex LH20 column chromatography, eluent: MeOH/H_2O (7:3), yielded compound **IV** as a fluffy white powder after lyophilization. LC–MS analysis indicated the presence of the desired product and a tri-i-butylphosphine–EA complex. The pure reduced tripeptide–EA conjugate was obtained after HPLC purification. R_f 0.3 (nBuOH/H_2O/AcOH, 15:3:2). ^{1}H NMR (D_2O): δ 0.86 (3H, t, CH_3EA), 1.55 (2H, m, CH_2EA), 1.74–1.92 (4H, m, $C_\beta H_2$, $C_\gamma H_2$) 2.4 (2H, m, $C_\delta H_2$) 2.85 (2H, dd, $C_\beta H_2$Cys), 3.0 (2H, m, CH_2EA), 3.74 (1H, d, C_αHEA) 3.78 (2H, d, CH_2Gly), 4.1 (1H, m, C_αHGlu), 4.65 (2H, s, CH_2EA), 6.9, 7.6 (2×1H, dd, EA aromatic). Mass spectrometry (ES-MS): m/e 596, $[M+H]^+$.

Boc-Glo(ONp)-OtBu (25). Boc-Ser(OtBu) (261.2 mg, 1.5 mmol) was dissolved in 20 mL DMF. Bis(4-nitrophenyl)carbonate (456 mg, 1.5 mmol) and DiPEA (259 μL, 1.5 mmol) were added, after which the resulting solution was stirred for 16 h at room temperature. EtOAc (100 mL) was added to the mixture, which was then repeatedly washed with saturated $NaHCO_3$ until the aq layer was no longer yellow. After drying and evaporation of the organic layer, the product was purified by silica gel column chromatography. The carbonate **25** was collected as a foam. Yield 582 mg (91%). R_f 0.85 (DCM/MeOH, 95:5). ^{1}H NMR ($CDCl_3$): δ 1.4 (9H, s, Boc), 1.5 (9H, s, tBu), 4.61 (3H, m, C_αH, $C_\beta H_2$), 5.62 (1H, d, $J=6.5$ Hz, NH), 7.4 (2H, aromatic) and 8.25 (2H, aromatic).

Boc-Glo[Cys(Acm)-Gly-OtBu]-OtBu (26). A solution of **4** (456 mg, 1.5 mmol) in 2.5 mL dioxane was added to a

solution of **25** (582 mg, 1.35 mmol) in 2.5 mL dioxane. The mixture was stirred at 80 °C overnight. After evaporation of the solvent, the residue was dissolved in 50 mL EtOAc. Washing with satd aq Na_2CO_3 was repeated until the aq layer was colorless. Silica gel column chromatography gave the pure tripeptide **26** as a foam. Yield 622 mg (78%). R_f 0.48 (DCM/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ 1.45 (27H, s, Boc + tBu), 2.0 (3H, s, CH_3 Acm), 2.8–3.0 (2H, m, $C_\beta H_2$Cys), 3.8–4.1 (2H, m, CH_2 Acm), 4.1–4.6 (6H, m, CH_2 Gly, C_αHCys C_αH, $C_\beta H_2$ Glo), 5.6 (1H, d, NH Boc), 6.2 (1H, d, NH Glo), 7.4 (2H, 2×NH, Acm and Gly amide). ^{13}C NMR ($CDCl_3$): δ 22.6 (CH_3 ACM), 27.6–28.0 (9×CH_3, Boc, tBu), 33.6 (C_β Cys), 40.4 (CH_2 Acm), 41.7 (CH_2 Gly), 53.7 (C_α Cys, C_α Glo), 65 (C_β Glo), 79.5–82.2 (3×C_{quart}, Boc, tBu), 155.1 (CO, Boc), 155.8 (CO, Glo), 168.4 (CO, Gly-tBu ester), 168.7 (CO, Acm), 170.7 (CO, Glo-tBu ester), 171.3 (CO, Cys)

(Boc-Glo[Cys-Gly-OtBu]-OtBu)$_2$ (**27**). Compound **26** (591 mg, 1 mmol) was dissolved in 7 mL of dry MeOH. A solution of I_2 (508 mg, 2 mmol) in 3 mL MeOH was added dropwise during 30 min. After 10 min at room temperature, the mixture was cooled to 0 °C and decolorized with 1 M aq $Na_2S_2O_3$ solution. The resulting mixture was diluted with 100 mL EtOAc and washed with 1M aq $Na_2S_2O_3$ solution and water. After drying and evaporation of the organic phase, the residue was dissolved in DCM/MeOH (1:1) and purified by Sephadex LH20 gelfiltration chromatography, using DCM/MeOH as eluent. The pure disulfide **27** was obtained as a white foam. Yield 494 ml (95%). R_f 0.51 (DCM/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ 1.45 (54H, s, Boc, tBu), 2.95 (4H, m, $C_\beta H_2$), 3.9 (4H, m, CH_2Gly), 4.3–4.5 (6H, C_αHGlo, $C_\beta H_2$Glo), 4.98 (2H, m, C_αHCys), 5.4 (2H, d, J = 8 Hz, NHBoc), 5.9 (2H, d, J = 8.2 Hz, NHGlo), 7.8 (2H, m, NHGly)

(H-Glo[Cys-Gly-OH]-OH)$_2$ (**28**). Disulfide **27** (494 mg, 0.48 mmol) was dissolved in 5 mL TFA/100μL H_2O. After 3 h at room temperature, the deprotected peptide was precipitated by addition of 15 mL ice-cold diethylether. The disulfide (TFA salt) **28** was used without further purification.

H-Glo[Cys(EA)-Gly-OH]-OH, VI. The deprotected disulfide **28** (296 mg, 0.48 mmol) was dissolved in 15 mL nPrOH/H_2O (2:1). Aqueous ammonia solution (25%) was added until pH = 8.5. After flushing with N_2, tri-*n*-butylphosphine (123 μL, 0.5 mmol) was added and the solution was stirred for 60 min at room temperature. A solution of ethacrynic acid (455 mg, 1.5 mmol) in 5 mL nPrOH was added. After adjusting the solution to pH 8 with 1 M NaOH, stirring was maintained for 4 h. The solvent was evaporated under reduced pressure, after which the residue was dissolved in water. The aqueous solution was extracted with EtOAc and purified by Sephadex LH20 column chromatography, using MeOH/H_2O (7:3) as eluent. Product **VI** was further purified by reversed phase HPLC to yield a fluffy white powder after lyophilization. R_f 0.35 (nBuOH/H_2O/AcOH, 15:3:2). ^{1}H NMR (D_2O): δ 0.9 (3H, t, CH_3EA), 1.55 (2H, m, CH_2EA), 2.9 (2H, dd, $C_\beta H_2$Cys), 3.0 (2H, m, CH_2EA), 3.75 (1H, d, C_αHEA) 3.9 (2H, d, CH_2Gly), 4.3–4.5 (3H, m, C_αHGlo, $C_\beta H_2$Glo), 4.65 (2H, s, CH_2EA), 4.9 (2H, m, C_αHCys), 6.9 and 7.6 (2×1H, dd, EA aromatic). Mass spectrometry (ES–MS) m/e 610, $[M-H]^-$ (Negative mode)

Stability towards γ-Glutamyl transpeptidase

The rate of degradation was determined by incubating 250 μM of the GS-EA analogues at 37 °C with 0.25 mg/mL bovine kidney γ-glutamyltranspeptidase in 0.1 M Tris–HCl buffer, pH = 7.4, supplemented with 0.1 mM EDTA. At selected time-intervals, a 100 μL sample was taken, heat-inactivated, and stored on ice until HPLC-analysis. Analysis was performed by RP-HPLC on an Alltech Platinum C18 column, using 50 mM ammonium acetate buffer pH = 3.8, supplemented with 10–20% acetonitrile (optimized for the various peptide analogues) as eluent. Disappearance of the parent peak and emergence of the corresponding dipeptide-EA conjugate was detected by UV absorbency at 270 nm. Remaining percentage of the parent compounds was determined as ratio of peak areas relative to the initial (t = 0 min) value. Results were corrected for non-peptidase mediated degradation of the ethacrynic acid conjugates.

Inhibition of rat liver cytosolic GSTs

Freshly isolated rat livers were emulsified in 0.1 M KCl using a Potter–Elvehjem homogeniser. After initial centrifugation for 30 min at 9000g, the resulting supernatant was subjected to a second centrifugation for 75 min at $10^5 g$. The cytosolic protein containing supernatant (S100) was used for the GST inhibition experiments. GST inhibition experiments were performed according to Habig et al., modified for a 96-well assay. All solutions were kept on ice until use. Various concentrations of the inhibitors were incubated at 37 °C with rat liver cytosol (1 μL, containing 12 mg of total protein) in sodium-phosphate buffer, pH 6.5, supplemented with 0.1 mM EDTA and 1 mM GSH. Reaction was started by adding 4 μL CDNB in ethanol (final concentration = 1mM, maximal ethanol content = 2% v/v). Formation of the GS-CDNB conjugate was followed at 340 nm for 5 min. Initial reaction velocities were corrected for the spontaneous (not GST catalyzed) reaction rates. Inhibition was determined as percentage of the non- inhibited reaction rate. Experiments were performed three times with duplicate measurements.

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