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SYNTHESIS AND INITIAL CHARACTERIZATION OF γ-L-GLUTAMYL-L-THIOTHREONYL-GLYCINE AND γ-L-GLUTAMYL-L-ALLO-THIOTHREONYLGLYCINE AS STERIC PROBES OF THE ACTIVE SITE OF GLYOXALASE I

Xiaofu Xie and Donald J. Creighton*

The Laboratory for Chemical Dynamics
Department of Chemistry and Biochemistry
University of Maryland Baltimore County
Baltimore, MD 21228

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The diastereomeric GSH derivatives γ-L-Glu-L-allo-thioThr-Gly (**6**) and γ-L-Glu-L-thioThr-Gly (**6a**) have been synthesized as specific probes of the steric environment near the cysteinyl residue of enzyme bound glutathionyl substrates. Experiments with glyoxalase I indicate that while **6a**-methylglyoxal thiohemiacetal is a substrate for the enzyme, **6**-methylglyoxal thiohemiacetal forms a tight-binding abortive complex with the active site ($K_i \simeq 100$ μM). Apparently, the small size of the cysteinyl C_β-H_s proton of the normal GSH-methylglyoxal thiohemiacetal substrate for glyoxalase I is a strict requirement for productive substrate binding. These compounds may provide a novel approach to the inhibition of GSH-dependent enzymes.

GSH (γ-L-Glu-L-Cys-Gly) has been identified as an essential cofactor for a myriad of vital metabolic processes within cells [1-3]. For this reason, numerous homologs and analogs of GSH have been synthesized for their potential pharmacological properties, and more specifically as probes of the structural features of the active sites of GSH-dependent enzymes [4]. In most cases, these derivatives involve changes in the γ-glutamyl or glycyl residues of GSH; less attention has been paid to altering the central cysteinyl residue most directly involved in catalysis by GSH-dependent enzymes.

*To whom correspondence should be addressed.

Abbreviations: GSH, glutathione; DCC, 1,3-dicyclohexylcarbodiimide; BOC, t-butyloxycarbonyl; Bzl, benzyl; Tsl, p-toluenesulfonyl; DMF, dimethylformamide; TEA, triethylamine; TFA, trifluoroacetic acid; BOCON, 2-(t-butyloxycarbonyloxyimino)-2-phenylacetonitrile;DSS,2,2-dimethyl-2-silapentane-5-sulfonate; Glx I, glyoxalase I; D, deuterium; FAB, fast atom bombardment; HPLC, high performance liquid chromatography.

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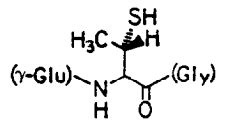
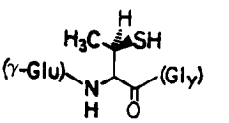
We report here the first synthesis of two novel GSH derivatives in which the C_β–H_s and C_β–H_r protons of cysteinyl have been alternately replaced by methyl groups to give **6** and **6a**, respectively.



6



6a

These compounds were specifically designed to be tested as alternate substrates for the GSH-dependent enzyme glyoxalase I (Glx I, EC 4.4.1.5) in order to probe the steric environment near the cysteinyl residue of bound GSH-methylglyoxal thiohemiacetal substrate. In addition, the aberrant structural features of **6** and **6a** were anticipated to potentially give rise to the formation of stable abortive complexes with the active site, thus, providing a novel approach to enzyme inhibition [5,6].

EXPERIMENTAL METHODS

Materials. GSH, glycine benzyl ester (p-toluenesulfonate salt), N-t-BOC-glutamate α-O-benzyl ester, and yeast glyoxalase I were purchased from Sigma and used without further purification. All other reagents used in the synthetic methods were Aldrich products. Methylglyoxal was synthesized by acid hydrolysis of the corresponding dimethylacetal [7]. p-Toluenesulfonyl chloride was recrystallized from petroleum ether.

N-t-BOC-L-threonylglycine benzyl ester (2) and its diastereomer (2a) were synthesized by DCC-coupling of glycine benzyl ester to N-t-BOC-L-threonine (1) and to N-t-BOC-L-allo-threonine (1a), respectively; the latter compound was prepared by reaction of L-allo-threonine with BOCON using a standard method [8]. Into anhydrous CH_2Cl_2 (40 mL) was dissolved 1 or 1a (1.73 g, 8 mmol) and glycine benzyl ester (p-toluenesulfonate salt) (2.66 g, 8 mmol). The reaction mixture was cooled on ice, TEA (8 mmol) and DCC (1.65 g, 8 mmol) were added, and the mixture was stirred for 2 h. After being warmed to room temperature, the mixture was stirred for an additional 20 h and stored at −10°C for 5 h. The precipitate (dicyclohexylurea) was removed by filtration and the solvent was removed from the filtrate in vacuo. The colorless oily residue was dissolved in ethyl acetate (200 mL), which was successively washed with 3 X 40 mL 1N HCl, 2 X 80 mL 1M $KHCO_3$, and 2 X 40 mL H_2O. The ethyl acetate phase was dried over anhydrous $MgSO_4$, concentrated to ~10 mL in vacuo, and brought to a cloud point with petroleum ether (~150 mL). After storage at −10°C for 2 days, the product was obtained as colorless needilic crystals. Recrystallization from the same solvent system afforded pure product: **2** (yield 72%; m.p. 60–62°); **2a** (yield 58%, m.p. 101–103°).

N-t-BOC O-Tosyl-L-threonylglycine benzyl ester (3) and its diastereomer (3a) were synthesized by tosylation of 2 and 2a, respectively. Into anhydrous pyridine (1.5 mL) was dissolved 2 or 2a (0.183 g, 0.5 mmol). The reaction mixture was cooled to −10°C, p-toluenesulfonyl chloride (0.257 g, 1.35 mmol) was added, and the mixture was stirred for 4 h. The solvent was removed in vacuo and the oily solid residue was triturated with several volumes of ice-cold water. The residue was then purified by reverse-phase HPLC (Whatman C-18; 22 mm i.d. X 50 cm) using methanol:water (4:1) as an eluting solvent: 3 (261 mL), 3a (271 mL). Evaporation of the solvent from the peak fractions afforded the desired products as colorless oils. 3 (yield 69%): H-1 80 MHz (D_3CCN:D_2O(1:0.6), DSS) – δ 1.28 (Thr-CH_3, d, J=6.3 Hz); δ 1.39 (t-butyl, s); δ 2.40 (Tsl-CH_3, s); δ 3.77 (Gly-CH_2, s); δ 4.24 (Thr-$C_\alpha H$, d, J=3.5 Hz); δ 4.95 (Thr-$C_\beta H$, m); δ 5.14

(benzyl-CH_2, s); δ 7.37 (benzyl ring-H, s); δ 7.39 (Tsl ring-H(ortho), d, J=7.3 Hz); δ 7.74 (Tsl ring-H(meta), d, J=8.1 Hz). **3a** (yield 39%): H-1 80 MHz (D_2O:D_3CCN(0.6:1), DSS) - δ 1.17 (Thr-CH_3, d, J=6.5 Hz); δ 1.38 (t-butyl, s); δ 2.43 (Tsl-CH_3, s); δ 3.91 (Gly-CH_2, s); δ 4.35 (Thr-$C_\alpha H$, d, J=4.7 Hz); δ 4.85 (Thr-$C_\beta H$, m,); δ 5.14 (benzyl-CH_2, s); δ 7.36 (benzyl ring-H, s); δ 7.43 (Tsl ring-H(ortho), d, J=8.4 Hz); δ 7.78 (Tsl ring-H(meta), d).

N-t-BOC S-benzyl L-allo-thiothreonylglycine benzyl ester (4) and its diastereomer (4a) were prepared from **3** and **3a** (respectively) by displacement of the tosyl functions with benzyl mercaptide ion. Into DMF (1 mL) under an argon atmosphere was dissolved **3** or **3a** (0.202 g, 0.4 mmol), followed by the addition of a freshly prepared solution of sodium benzyl mercaptide (0.8 mmol) dissolved in DMF (0.3 mL). The reaction mixture was stirred at room temperature for 2 h; water (0.5 mL) was added; and the mixture was brought to pH 7 with 0.1N HCl. The mixture was then extracted with 3 X 5 mL ethyl acetate. The ethyl acetate phase was successively washed with 0.1 N HCl (3 x 5 mL), 5% aqueous $NaHCO_3$ (3 x 5 mL), and H_2O (3 x 5 mL). The solvent from the ethyl acetate phase was removed *in vacuo*, and the oily residue purified by reverse phase HPLC as described above using methanol:water (4:1) as an eluting solvent: **4** (309 mL); **4a** (333 mL). Evaporation of the solvent from the peak fractions afforded the final products as colorless oils: **4** (yield 52%): H-1 80 MHz (D_3CCN:D_2O(1:0.6), DSS) - δ 1.09 (thioThr-CH_3, d, J=7.2 Hz), δ 1.42 (t-butyl, s); δ 3.20 (thioThr-$C_\beta H$, m); δ 3.77 (S-benzyl-CH_2, s); δ 3.98 (Gly-CH_2, s); δ 4.31 (thioThr-$C_\alpha H$, d, J=5.0 Hz); δ 5.15 (O-benzyl-CH_2, s); δ 7.32 (S-benzyl ring-H, s); δ 7.37 (O-benzyl ring-H, s). **4a** (yield 55%): H-1 80 MHz (D_3CCN:D_2O(1:0.6), DSS) - δ 1.21 (thioThr-CH_3, d, J=7.1 Hz); δ 1.41 (t-butyl, s); δ 3.16 (thioThr-$C_\beta H$, m); δ 3.75 (S-benzyl-CH_2, s); δ 3.99 (Gly-CH_2, s); δ 4.19 (thioThr-$C_\alpha H$, d, J=5.4 Hz); δ 5.15 (O-benzyl-CH_2, s); δ 7.30 (S-benzyl ring-H, s); δ 7.37 (O-benzyl ring-H, s).

N-t-BOC O,S-dibenzyl-γ-L-glutamyl-L-allo-thiothreonyl glycine benzyl ester (5) and its diastereomer (5a). The t-BOC groups were removed from **4** and **4a** (95 mg, 0.2 mmol) by incubation in TFA (2 mL) at room temperature for 0.5 h. The solvent was removed *in vacuo* to afford the trifluoroacetate salts of the deblocked dipeptides. These were then converted to their free base forms by incubation with 2 equivalents of TEA in anhydrous THF (0.5 mL) at 0°C for 0.5h. The triethylammonium trifluoroacetate was removed by filtration, and the solvent was evaporated from the filtrate to give the free base forms of the dipeptides as light yellow oils (yields 95-100%). The oil was then dissolved in methylene chloride (3 mL) containing N-t-BOC glutamic acid α-O-benzyl ester (258 mg, 0.76 mmol), followed by the addition of DCC (187 mg, 0.9 mmol). After cooling to 0°C, TEA (0.64 mmol) was added, and the mixture was stirred for 1 h at 0°C and 20 h at room temperature. After storage at -10°C for 5 h, the dicyclohexylurea was removed by filtration, and the solvent was removed from the filtrate *in vacuo*. The resultant oily residue was dissolved in ethyl acetate (30 mL), which was successively washed with 1 N HCl (3 x 10 mL), 2.5% aqueous $NaHCO_3$ (3 x 10 mL), and water (3 x 10 mL). The ethyl acetate phase was dried over anhydrous Na_2SO_4, and the solvent removed to give the crude product as a colorless oil. Analytically pure product was obtained by reverse phase HPLC as described above using methanol:water (4:1) as an eluting solvent: **5** (520 mL); **5a** (634 mL). Removal of the solvent from the peak fractions gave the pure products as colorless oils: **5** (yield 75%): H-1 80 MHz (D_3CCN:D_2O(1:0.6), DSS) - δ 1.11 (thioThr-CH_3, d, J=7.2 Hz); δ 1.36 (t-butyl, s); δ 3.74 (S-benzyl-CH_2, s); δ 3.97 (Gly-CH_2, s); δ 5.15 (O-benzyl-CH_2, s); δ 7.30 (S-benzyl ring-H, s); δ 7.37 (O,O-dibenzyl ring-H, s). FAB mass spectrum: m/z (rel. intensity) - 692 (38%) $[M+H]^+$; 592 (100%) $[M_2\text{-}M\text{-}[C(CH_3)_3COCO] + H]^+$; 502 (33%) $[M_3\text{-}M_2\text{-}[CH_2C_6H_5] + H]^+$. **5a** (yield 34%): H-1 80 MHz (D_3CCN:D_2O(1:0.6), DSS) -δ 1.11 (thioThr-CH_3, d, J=6.2 Hz); δ 1.36 (t-butyl, s); δ 3.75 (S-benzyl-CH_2, s); δ 3.98 (Gly-CH_2, s); δ 5.15 (O-benzyl-CH_2, s); δ 7.29 (S-benzyl ring-H, s); δ 7.37 (O,O-dibenzyl ring-H, s).

γ-Glutamyl-L-allo-thiothreonyl glycine (6) and its diastereomer (6a). Removal of the benzyl functions from **5** and **5a** (35.5 mg, 0.05 mmol) first involved dissolution in liquid NH_3 (30 mL). Into this stirring solution was inserted the end of a 50 μL glass capillary pipet filled with ~15 mg of sodium metal. The sodium metal was allowed to dissolve in the reaction mixture until a blue color persisted for at least 30 s. Ammonium chloride (35 mg) was added and the solvent

allowed to evaporate. The residue was dissolved in 20% acetic acid in water (1 mL), filtered, and desalted on a Sephadex G-10 column (1 X 70 cm) using argon saturated water containing the sodium salt of ethylenediaminetetraacetate (10 μM) as an eluting solvent. The product fractions were pooled, lyophilized, and the resultant residues incubated in TFA for 30 min. Evaporation of the TFA gave the crude products as colorless oils. Analytically pure product was obtained by reverse phase HPLC (Waters C18; 7.8 mm i.d. x 30 cm) using 0.25% aqueous acetic acid as an eluting solvent: **5** (30 mL); **5a** (35 mL). Evaporation of the solvent from the peak fractions gave the final products as colorless oils: **6** (yield 13.7%): H-1 500 MHz (D_2O, DSS) - δ 1.345 (thioThr-CH_3, d, J=7.0 Hz); δ 2.151 (Glu-$C_\beta H_2$, q); δ 2.551 (Glu-$C_\gamma H_2$, m); δ 3.419 (thioThr-$C_\beta H$, p); δ 3.777 (Glu-$C_\alpha H$, t); δ 3.891 (Gly-CH_2, s); δ 4.539 (thioThr-$C_\alpha H$, d, J=6.0 Hz). FAB mass spectrum: m/z (rel. intensity) - 322 (31%) $[M+H]^+$; 307 (100%) $[M-[CH_3] + H]^+$. **6a** (yield 2.5%): H-1 500 MHz (D_2O, DSS) - δ 1.327 (thioThr-CH_3, d, J=7.0 Hz); δ 2.159 (Glu-$C_\beta H_2$, q); δ 2.590 (Glu-$C_\gamma H_2$, m); δ 3.578 (thioThr-$C_\beta H$, p); δ 3.769 (Glu-$C_\alpha H$, t); δ 3.769 (Gly-CH_2, s); δ 4.590 (thioThr-$C_\alpha H$, d, J=5.0 Hz). FAB mass spectrum: m/z (rel. intensity) - 322 (33%) $[M+H]^+$, 307 (100%) $[[M-CH_3] + H]^+$. 289 (81%) $[[M-SH] + H]^+$.

Analytical Methods. NMR spectra were taken on IBM NR-80 or GE GN-500 NMR spectrometers. The NMR spectral assignments for the compounds reported here are as expected on the basis of comparisons with published chemical shift and coupling constant data for amino acids and peptides [9] and by comparison with a previous NMR study of GSH in aqueous solution [10]. FAB mass spectra were taken on a JEOL HX 110/HX 110 tandem four sector mass spectrometer. Kinetic measurements were obtained with a Gilford Response spectrophotometer. The dissociation constants (K_{diss}) of the methylglyoxal thiohemiacetals formed with GSH and with **6** were determined by a published spectrophotometric method [11]; $K_{diss} = [M]_t[\text{GSH or } 6]/[\text{thiohemiacetal}]$, where M_t is equal to the sum of the free aldehyde and hydrated forms of methylglyoxal.

RESULTS AND DISCUSSION

Synthesis. The strategy used to assemble **6** and **6a** (Figure 1) was formulated on the basis of earlier reports that benzyl mercaptide ion and thioacetate ion will react with O-tosyl L-serine peptides to give the respective S-benzyl and S-acetyl L-cysteine peptides via S_N2 displacement reactions [12]. Thus, the blocked forms of L-threonylglycine (**2**) and L-allo-threonylglycine (**2a**) were synthesized by standard methods [13], followed by treatment with toluene sulfonyl chloride to give **3** and **3a**. Central to the synthetic strategy is the S_N2 displacement of the tosyl functions from **3** and **3a** by benzyl mercaptide ion to give the respective S-benzyl dipeptides (**4** and **4a**) in fair yields (50-60%). That the displacement reactions proceed with complete inversion of configuration at C_β is supported by reverse-phase chromatographic analysis of the respective product mixtures in which the preparation of **4** was found to be free of **4a** and the preparation of **4a** was free of **4**. Removal of the t-BOC functions from **4** and **4a** using trifluoroacetic acid, followed by DCC-coupling with N-t-BOC glutamic acid α-O-benzyl ester afforded the blocked tripeptides **5** and **5a** with yields of 75 and 38%, respectively. The target tripeptides (**6** and **6a**) were then obtained using standard methods of deprotection.

The sequence of steps and the nature of the reagents used to synthesize **6** and **6a** were constrained by two observations. First, early attempts to synthesize

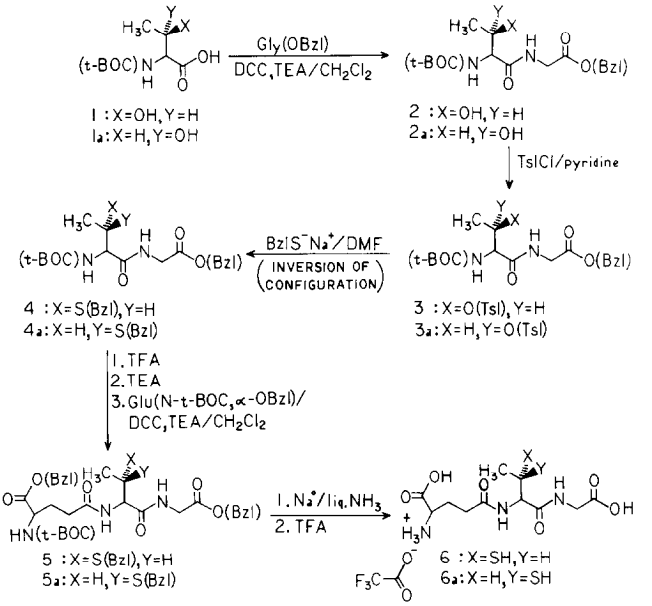
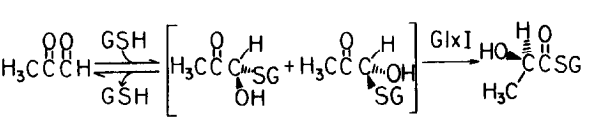


Figure 1. Synthetic route to γ-L-glutamyl-L-allo-thiothreonylglycine (**6**) and to γ-L-glutamyl-L-thiothreonylglycine (**6a**).

6 directly from γ-L-glutamyl-L-threonylglycine were unsuccessful because of the inability to tosylate the blocked version of this tripeptide. However, tosylation of the dipeptides (**2** and **2a**) proceeded with reasonable yields. Second, the use of thioacetate ion in place of benzyl mercaptide ion was unsatisfactory because of the inability to couple S-acetyl N-t-BOC thiothreonylglycine α-O-benzyl ester with N-t-BOC-glutamic acid α-O-benzyl ester using DCC without loss of the S-acetyl function [12].

Kinetic studies. Glx I was selected for initial testing with **6** and **6a** because inhibitors of this enzyme have attracted attention as possible antineoplastic agents [4]. This enzyme normally functions to convert cytotoxic methylglyoxal in cells to S-D-lactoylglutathione; the direct substrates for the enzyme are the two diastereomeric thiohemiacetals formed by preequilibrium addition of GSH to methylglyoxal [6,14], eq. 1.



(1.

Importantly, the catalytic conversion of bound substrate to bound product may involve significant changes in the side chain conformation of the cysteinyl residue of substrate, on the basis of paramagnetic nuclear relaxation [5] and enzyme inhibitor studies [6]. Therefore, we reasoned that the **6**- and/or **6a**-methylglyoxal thiohemiacetals might serve as powerful dead-end inhibitors of Glx

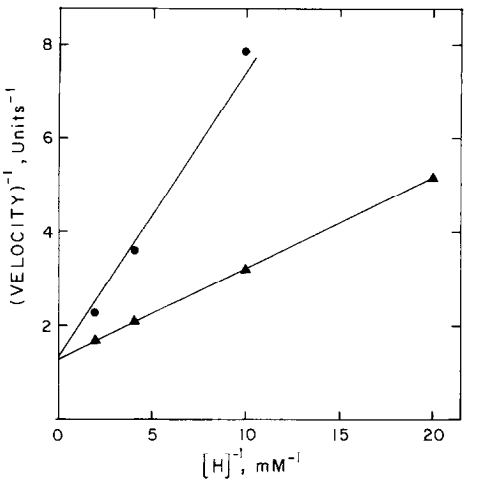


Figure 2. Reciprocal plot of glyoxalase I activity versus the concentration of GSH-methylglyoxal thiohemiacetal (H) in the presence (●) and in the absence (Δ) of **6a**-methylglyoxal thiohemiacetal (H') (0.22 mM); phosphate buffer (25 mM, pH 7), 25°C; ionic strength = 0.1 M using NaCl. The concentrations of free GSH and H' were kept constant at 0.2 mM and 0.22 mM (respectively) for each kinetic run by varying the total concentrations of GSH, methylglyoxal and **6a**, on the basis of K_{diss} (H) = 6.9 mM and K_{diss} (H') = 26.6 mM.

I, provided that the side-chain mobilities of the thiothreonyl and allo-thiothreonyl residues are restricted by the presence of bulky methyl groups at C_β. Experimentally, **6a** proved to be an alternate substrate for Glx I, on the basis of the observation that the initial rate of product formation (ΔOD_{240}) using **6a** as a substrate was ~10% of that using GSH under conditions where [**6a** or GSH] = 0.12 mM and [methylglyoxal] = 0.10 mM. In contrast, the thiohemiacetal formed with **6** did not show detectable activity with the enzyme, but rather appears to form an abortive complex with the active site. This conclusion is based on the observation that the extent of inhibition of Glx I by fixed concentrations of **6**-methylglyoxal thiohemiacetal in the presence of variable concentrations of GSH-methylglyoxal thiohemiacetal substrate is consistent with competitive inhibition ($K_i \simeq 100\ \mu M$) (Figure 2). Thus, the steric environment of the active site has sufficient latitude to bind the thiohemiacetals formed from both **6** and **6a**. Nevertheless, the small size of the cysteinyl C_β-H_s proton of the normal thiohemiacetal substrate appears to be a strict requirement for productive substrate binding.

CONCLUSIONS: The results of the studies reported here indicate that the two GSH analogs, **6** and **6a**, should be useful probes of the steric environments of GSH-dependent enzymes. Moreover, these molecules provide a potentially novel approach to enzyme inhibition.

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