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Synthesis of *S*-alkyl L-homocysteine analogues of glutathione and their kinetic studies with γ-glutamyl transpeptidase

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Abstract—A series of *S*-alkyl L-homocysteine analogues of glutathione was synthesized with varied oxidation state of the sulfur and tested for inhibition of rat kidney γ-glutamyl transpeptidase (GGT). The strong selectivity of the enzyme with respect to the sulfur oxidation state reveals important information for the development of powerful competitive inhibitors.
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γ-Glutamyl transpeptidase (GGT, EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme bound to the extracellular membrane of cells. It can be found in mammals,[1] bacteria[2] and plants[3] and is present in high concentrations in kidney, liver and brain[1]. It has been implicated in cellular detoxification processes, leukotriene biosynthesis, anti-cancer prodrug activation and physiological disorders including Parkinson's disease[4] and perturbation of apoptosis.[5,6] This enzyme also plays a key role in the metabolism of glutathione (GSH), its in vivo substrate, by catalyzing its cleavage between the γ-glutamyl and Cys-Gly moieties. In the first step of its catalytic cycle, GGT is acylated by transfer of a γ-glutamyl moiety from a donor substrate.[7] This acyl group is then transferred during the deacylation step to acyl acceptor substrates such as α-amino acids and peptides (transamidation), or to a water molecule (hydrolysis). The important residues responsible for catalysis by mammalian GGT are not well known. The catalytic nucleophile of bacterial GGT has been identified by mass spectrometry as Thr-391, the *N* terminal residue of the small subunit. This residue is conserved in all GGTs, suggesting that GGT could be a member of the Ntn-hydrolase family.[8]

In the course of our previous investigation of the mechanism of acylation of GGT, we reported the discovery of *S*-alkyl sulfoxides as a new class of reversible GGT inhibitors, proposing that they resemble GSH, the in vivo substrate. Testing of these analogues revealed a promising competitive inhibitor (L-SPG, Fig. 1, $n = 1$).[9] Herein we report the synthesis of a series of *S*-alkyl L-homocysteine GSH analogues, wherein the sulfur is present in three distinct oxidation states: as a thioether, a sulfoxide and the corresponding sulfone (Fig. 1). Inhibition constants are reported for these sulfur derivatives for the GGT-mediated reaction of the chromogenic donor substrate L-glutamic acid γ-*p*-nitroanilide (L-GPNA), with the efficient acceptor substrate Gly-Gly. The affinity of these *S*-alkyl inhibitors can be compared with the corresponding *S* methyl (i.e. L-Met) derivatives at the same oxidation state.

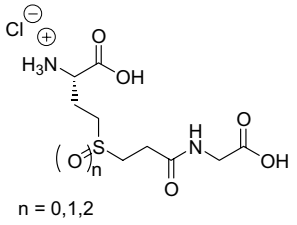

Figure 1. *S*-Alkyl L-homocysteine analogues of GSH, studied herein.

Scheme 1 depicts the synthesis of a thioether analogue of GSH (L-TEPG, **4**). The Boc-L-homocysteine methyl ester was prepared in three steps from L-homocystine as reported in the literature.[9] The first step from the L-homocysteine derivative is the condensation with bromide **1**, previously synthesized in one step according to a literature procedure,[9] to obtain **2**. The next step is

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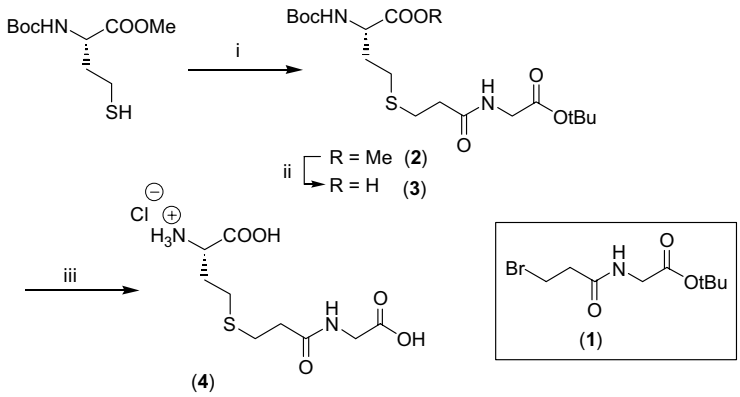


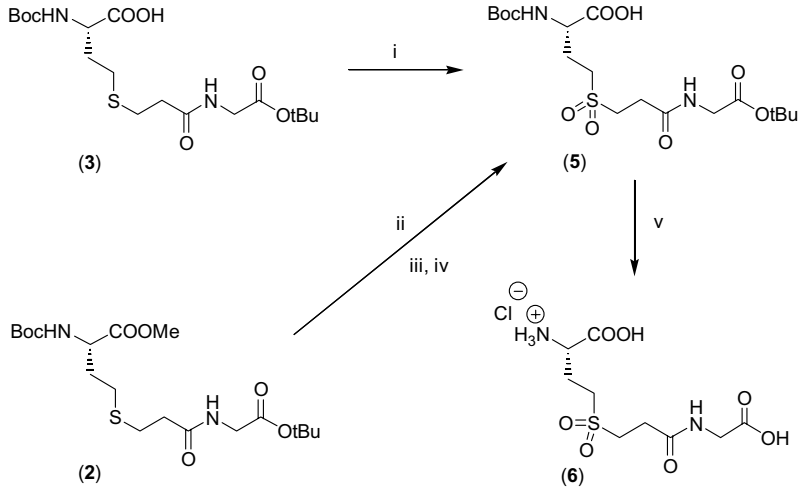
Scheme 1. Synthesis of L-TEPG (**4**). Reagents and conditions: (i) K_2CO_3, (**1**), DMF, 71%; (ii) NaOH, THF/H_2O; (iii) (a) TFA, CH_2Cl_2 at 0 °C; (b) HCl (0.1 N), 100% (two steps).

the hydrolysis of the methyl ester to provide **3** and removal of the Boc protecting group and *tert*-butyl ester with TFA to give the desired compound **4** with a high overall yield.

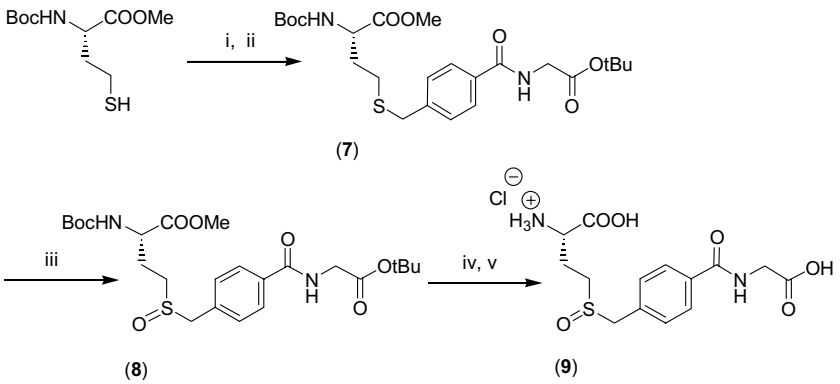
The sulfone GSH analogue (L-SnPG, **6**) shown in Scheme 2 was synthesized from compound **3**. Sulfone **5** was obtained directly through the oxidation of the *S* alkyl L-homocysteine derivative **3** by hydrogen peroxide in presence of acetic acid with a modest final yield of 51%. In an attempt to improve this yield, a second method was tried, involving the initial oxidation of **2** with sodium periodate to obtain the sulfoxide derivative. All attempts to push the reaction further to obtain the sulfone gave no further disappearance of the starting material, even with heating. Thus the sulfoxide intermediate was oxidized further using hydrogen peroxide in acetic acid with heating. After two days, the desired sulfone was obtained in 53% yield over these two steps. Subsequent hydrolysis of the methyl ester in alkaline media provided **5**, followed by removal of the Boc protecting group and the *tert*-butyl ester by the standard method (TFA) to obtain sulfone **6**.

Scheme 3 describes the synthesis of an aryl analogue (L-STG, **9**) of the previously synthesized[9] sulfoxide compound (L-SPG). An aromatic moiety was introduced in the place of the propyl moiety present in L-SPG, since hippurate (benzoylglycine) is known to be one of the best activators of the hydrolysis reaction catalyzed by GGT. This activator has been proposed to be bound at the same place as the Cys-Gly moiety of GSH, where we propose that the propylglycine moiety of L-SPG may be bound. Analogue **9** was thus designed combining recognition elements of donor substrate L-GPNA, the sulfoxide inhibitor LSPG and the activator hippurate.

The first step in the synthesis of L-STG is the condensation of the L-homocysteine derivative with commercially available β-bromotoluic acid. The aryl carboxylate derivative was then condensed with glycine *tert*-butyl ester·HCl in the presence of the coupling agent TBTU to give the thioether derivative **7**. Subsequent oxidation of **7** with $NaIO_4$ provided sulfoxide **8** in good yield. The remaining steps represent the straightforward deprotection of the molecule with sodium hydroxide to remove the α-methyl ester, followed by removal of the



Scheme 2. Synthesis of L-SnPG (**6**). Reagents and conditions: (i) H_2O_2, AcOH, THF, 51%; (ii) $NaIO_4$, THF/H_2O, 91%; (iii) H_2O_2, AcOH, MeOH, 58%; (iv) NaOH, THF/H_2O, 81%; (v) (a) TFA, CH_2Cl_2 at 0 °C; (b) HCl (0.1 N), 100%.



Scheme 3. Synthesis of L-STG (**9**). Reagents and conditions: (i) β-bromotoluic acid, K_2CO_3, DMF; (ii) glycine *t*-butyl ester·HCl, DIEA, TBTU, CH_3CN, 44% (two steps); (iii) $NaIO_4$, THF/H_2O, 92%; (iv) NaOH, THF/H_2O, 94%; (v) (a) TFA, CH_2Cl_2 at 0 °C; (b) HCl (0.1 N), 93%.

Boc protecting group and *tert*-butyl ester with TFA to give the desired product **9**.

All synthetic reactions were followed by TLC and the synthetic intermediates were characterized by ^1H and ^{13}C NMR and MS. L-GPNA was synthesized as previously described.[10] Compounds (**4**),[11] (**6**)[12] and (**9**)[13] were selected for kinetic evaluation[14] as inhibitors of GGT, purified from rat kidney as previously described.[15] Parameters from Michaelis–Menten plots were re-plotted against the concentrations of each inhibitor. The K_i of sulfone **6** was calculated from its IC_{50} value using the K_M of L-GPNA as 590 μM and presuming competitive inhibition. The results from these tests are reported in Table 1.

Compounds **4**, **6** and **9** may be categorized as analogues of L-methionine or GSH, depending on the alkyl substituent at the δ-position, and according to the oxidation state of the sulfur. L-TEPG (**4**) is thus a GSH analogue wherein the γ-amide bond has been replaced with a thioether functionality. Inhibition studies of this compound demonstrate competitive inhibition with a K_i of (520 ± 40) μM, around 10 times higher than that of the corresponding sulfoxide (L-SPG, $K_i = (53 \pm 3)$ μM).[9] By comparison with L-methionine, the affinity of LTEPG is around 50-fold greater due to the pendant propylglycine moiety, which resembles the Cys-Gly moiety of GSH, the in vivo substrate of GGT. This moiety is evidently critically important to favourable binding interactions with the donor substrate binding site of the enzyme. Other analogues of GSH have been synthesized[17] where the isopeptide bond has been replaced with a dialkyl amino linkage, and these have also demonstrated good inhibition.

Table 1. Type of inhibition and K_i for each compound tested in presence of rat GGT at pH 8.0 and 37 °C

Compounds	Type of inhibition	K_i (mM)
L-Methionine	Competitive	26.9[a]
L-Methionine sulfoxide	Competitive	5.9 ± 0.2[b]
L-Methionine sulfone	—	N/o[b,c]
L-TEPG (**4**)	Competitive	0.52 ± 0.04
L-SPG	Competitive	0.053 ± 0.003[b]
L-SnPG (**6**)	—	3.5 ± 0.6[d]
L-STG (**9**)	Competitive	0.23 ± 0.03

[a] See Ref. 16.
[b] See Ref. 5.
[c] N/o: No inhibition observed up to 20 mM.
[d] K_i calculated from IC_{50}, presuming competitive inhibition.

In order to probe the importance of the propylglycine moiety, it is also instructive to compare sulfone **6** (L-SnPG) and L-methionine sulfone. The methionine derivative is unable to inhibit GGT but L-SnPG, bearing the propylglycine moiety, shows inhibition for which a K_i of (2.9 ± 0.6) mM was calculated. This calculation was made assuming the inhibition was competitive, since the structure of inhibitor **6** is very similar to those of thioether **4** and sulfoxide (L-SPG) derivatives, which are known competitive inhibitors against L-GPNA. The sulfonamide analogue of L-SnPG, which could inhibit GGT irreversibly, has been synthesized, but no kinetic data has been given as proof of the efficacy of this molecule.[18] Compared to the sulfoxide, the second S=O double bond in the sulfone may lead to a loss of affinity for the donor binding site, perhaps due to steric hindrance. Finally, the favourable effect of the propylglycine moiety is further confirmed by comparing L-methionine sulfoxide with L-SPG; the GSH analogue is 100 times more efficient as a competitive inhibitor than the corresponding methionine derivative.

As discussed above, hippurate is one of the best activators of GGT-mediated hydrolysis, purportedly binding in the same place as the Cys-Gly moiety of GSH,[19] so a benzoylglycine moiety was employed in sulfoxide derivative **9** as a mimic of the Cys-Gly fragment. It was found that L-STG (**9**) acts as a competitive inhibitor; V_{max} did not vary as a function of inhibitor concentration (data not shown), but K_M varied by the factor of $(1 + \frac{[I]}{K_i})$, giving a K_i of (225 ± 30) μM (Fig. 2). This aromatic derivative is thus a less efficient inhibitor than the sulfoxide derivative bearing a propylglycine moiety (L-SPG). However, its affinity may still be better than GSH, the natural substrate of GGT, whose K_M value is around 500 μM.[20] This demonstrates that the sulfoxide group is nevertheless a good pharmacophore for the inhibition of GGT as a substitute for the labile γ-glutamyl amide bond of GSH.

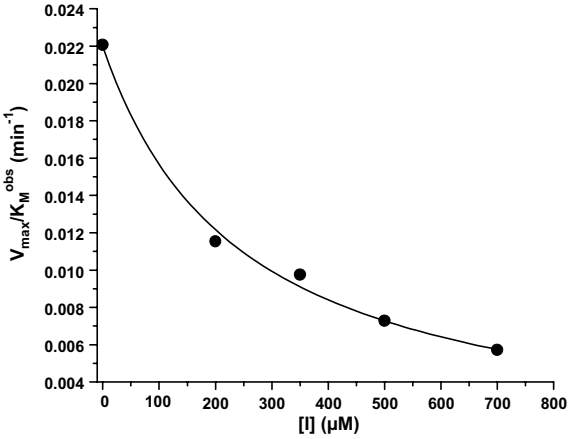


Figure 2. Replot for the competitive inhibition of L-GPNA by L-STG (**9**).

The inhibition efficiency displayed by sulfoxides compared to thioethers or sulfones may be due to their resemblance of the tetrahedral intermediate formed during the acylation of GGT. Based on mutagenesis experiments, two adjacent serines, conserved in all GGTs, have been implicated in important active site binding roles, including the stabilization of the oxyanion of the tetrahedral intermediate of the acylation step.[1] It is conceivable that sulfoxide L-SPG could be bound and stabilized in the same fashion (Fig. 3). Further interactions could also exist between the partially positively charged sulfur of the inhibitor and the threonine residue that is the probable active site nucleophile.

While no three-dimensional structure of GGT presently exists to verify this hypothetical binding mode, other lines of evidence suggest that these interactions are highly spatially orientated. We have previously demonstrated that the interaction between GGT and sulfoxide inhibitors is remarkably stereospecific with respect to the configuration of the δ stereocentre, in that one diastereoisomer inhibits with a K_i of 3.4 mM, whereas the other diastereoisomer is not recognized.[9] Following a literature protocol,[21] we have isolated one of the two diastereomers by crystallization as its picrate salt and determined its absolute configuration by X-ray diffraction (Fig. 4).[22] Subsequent testing of the optically pure compound confirmed that the isolated S_CS_S diastereomer of L-Met sulfoxide is specifically bound by GGT.

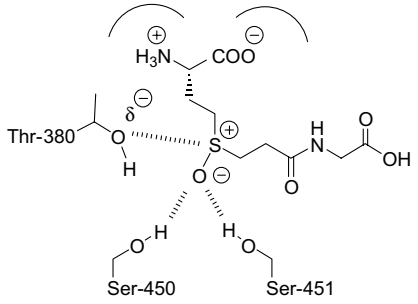


Figure 3. Possible interactions between the inhibitor (L-SPG) and the active site of rat kidney GGT.

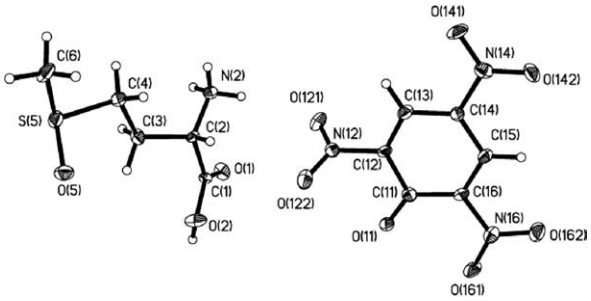


Figure 4. X-ray structure of the picrate salt of the inhibitory S_CS_S methionine sulfoxide.

In summary, we have synthesized a series of *S*-alkyl L-homocysteine derivatives with different oxidation states of the sulfur. The sulfoxide derivative is 10 times more efficient than the thioether compound, which is in turn 10 times more efficient than the corresponding sulfone. Further comparison suggests that the propylglycine moiety contributes significantly to its affinity for rat kidney GGT, providing in the best case (L-SPG) a low micromolar K_i value. It is also important to note that these sulfoxides were tested as 50/50 mixtures of *S* diastereoisomers, whereas we have confirmed with the L-Met derivative that only the S_S diastereoisomer is recognized. This demonstrates the importance of stereospecific synthesis of the S_S diastereoisomer for future work.

Acknowledgements

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11. (**4**) Gummy solid: $[\alpha]_D$ − 3.0 (*c* 0.32, MeOH); ^{1}H NMR (D_2O, 400 MHz) δ 2.07–2.20 (m, 2H), 2.54 (t, $J = 7.0$ Hz, 2H), 2.65 (t, 2H, $J = 7.1$ Hz, 2H), 2.75 (t, $J = 6.8$ Hz, 2H), 3.90 (s, 2H), 4.13 (m, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 26.7, 26.8, 29.7, 35.5, 41.3, 51.9, 141.9, 173.6, 175.3; *m/z* 265.1 (MH^+, $C_9H_{17}N_2O_5S$ requires 265.0858).
12. (**6**) Gummy solid: $[\alpha]_D$ − 3.7 (*c* 0.28, MeOH); ^{1}H NMR (D_2O, 400 MHz) δ 2.36–2.47 (m, 2H), 2.85 (t, $J = 7.1$ Hz, 2H), 3.39–3.52 (m, 2H), 3.56 (t, $J = 7.1$ Hz, 2H), 3.97 (s, 2H), 4.21 (t, $J = 6.6$ Hz, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 22.5, 27.7, 41.5, 48.3, 48.7, 51.5, 170.9, 172.7, 173.6; *m/z* 297.0 (MH^+, $C_9H_{17}N_2O_7S$ requires 297.0756).
13. (**9**) Gummy solid: $[\alpha]_D$ − 4.4 (*c* 0.32, MeOH); ^{1}H NMR (D_2O, 400 MHz) δ 2.33 (m, 2H), 2.93–3.08 (m, 2H), 4.11 (s, 2H), 4.15–4.22 (m, 1H + 1H), 4.27 (dd, $J = 13.2$ Hz, $J = 3.0$ Hz), 7.40 (d, $J = 8.1$ Hz, 2H), 7.75 (d, $J = 8.1$ Hz, 2H); ^{13}C NMR (CD_3OD, 75 MHz) δ 24.1 (d, mixture 50/50 of diastereoisomers), 42.3, 46.2 (d), 52.2 (d), 56.2 (d), 128.5, 131.6, 133.6, 134.2, 171.0, 171.4, 174.1; *m/z* 343.0964 (MH^+, $C_{14}H_{19}N_2O_6S$ requires 343.0948).
14. Reactions were initiated by the addition of 3.38 mU of GGT. Liberated *p*-nitroaniline was detected spectrophotometrically at 410 nm ($\varepsilon = 8800\ M^{-1}\ cm^{-1}$) on a Cary 100 Bio spectrophotometer. For inhibition studies, different concentrations (67–1400 μM) of LGPNA and a saturating concentration (20 mM) of Gly-Gly were used in the presence of varying concentrations (0–1 mM) of compounds L-TEPG (**4**) and L-STG (**9**) in 0.1 M Tris–HCl pH 8.0. An IC_{50} value was found with different concentrations (0–10 mM) for sulfone (**6**) in the presence of a fixed concentration of LGPNA (400 μM) and a saturating concentration (20 mM) of Gly-Gly. Blank experiments were done without enzyme. K_i values were determined by re-plots of V_{max}/K_M^{obs} versus [*I*] analyzed by Origin 6.0 curve-fitting software.
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22. Absolute configuration was established by structure determination using a stereocentre reference of known absolute configuration and confirmed by anomalous dispersion effects (sulfur atom) in diffraction measurements on the crystal. The Flack parameter refined to 0.07(6) and the crystallographic data were deposited at the Cambridge Crystallographic Data Centre as CCDC 236529.