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Glutathione-like Tripeptides as Inhibitors of Glutathionylspermidine Synthetase. Part 1: Substitution of the Glycine Carboxylic Acid Group

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Abstract—Glutathionylspermidine synthetase/amidase (GspS) is an essential enzyme in the biosynthesis and turnover of trypanothione and represents an attractive target for the design of selective anti-parasitic drugs. We synthesised a series of analogues of glutathione (L-γ-Glu-L-Leu-Gly-X) where the glycine carboxylic acid group (X) has been substituted for other acidic groups such as tetrazole, hydroxamic acid, acylsulphonamide and boronic acid. The boronic acid appears the most promising lead compound (IC_{50} of 17.2 μM).

The development of chemotherapy for the treatment of parasitic diseases such as sleeping sickness, Chagas disease and leishmaniasis has been hindered by the close similarities between parasite and host metabolism. However, one major difference occurs in the biochemistry of defence mechanisms against oxidative damage.[1]

Parasitic protozoa of the order Kinetoplastida are protected against damage by oxidants and toxic heavy metals by a unique thiol-redox cycling.[2] This system uses trypanothione [N^1,N^8-bis(glutathionyl)spermidine] and is analogous to the glutathione system that operates in humans and almost all other aerobic organisms. However, it offers a number of distinct features that may be exploited for selective attack. Trypanothione is the pivotal compound in a concerted cascade of reducing equivalents from NADPH as initial donor to a peroxide acceptor, requiring three distinct enzymes, specific to the Kinetoplastida: trypanothione reductase, tryparedoxin and tryparedoxin peroxidase. Several inhibitors of trypanothione reductase have been developed and the enzyme has been validated as a target for the design of trypanocidal compounds. The properties of trypanothione metabolism as a drug target have been reviewed.[3]

The biosynthesis of trypanothione from glutathione and spermidine is catalysed by glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS).[4,5] As carbon–nitrogen ligases, both enzymes use ATP and are proposed to form an acyl phosphate at the glycine carboxylate of glutathione, activating it for nucleophilic attack by the primary amines of spermidine.

GspS also contains an amidase domain, hydrolysing glutathionylspermidine into glutathione and spermidine. Similar to other ATP-dependent ligases, phosphonates,[6,7] phosphonamidates,[7] and phosphinates[8] derived from glutathione-like peptides are inhibitors of GspS from *Crithidia fasciculata* and the analogous enzyme from *Escherichia coli*. These compounds are thought to behave as substrate analogues or mimic the proposed tetrahedral intermediate formed during the attack of spermidine on the acyl phosphate.

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The results obtained with glutathione-derived tripeptidylphosphonic acids such as **1** (K_i of 60 μM-linear non-competitive inhibition) on the isolated enzyme from *C. fasciculata*[6] prompted us to start the synthesis and evaluation of related analogues in which the C-terminal carboxylic acid group is substituted with other acidic groups (Fig. 1).

We confined the current series to L-γ-Glu-L-Leu tripeptides, since we knew from previous substrate-selectivity studies that the γ-glutamyl moiety is essential for recognition by GspS and cannot be omitted. L-Leu was the best substitute for L-Cys in the phosphonopeptide inhibitors and was used also in this series.[6] Hence, tripeptide L-γ-Glu-L-Leu-Gly (**2**) was used as lead peptide. Carboxylic substitutes were acidic groups such as in tetrazole (**3**), boronic acid (**4**), acylsulphonamide (**5**) and hydroxamic acid (**6**) and non-acidic groups such as amide (**7**) and a carboxylic ester (**8**).

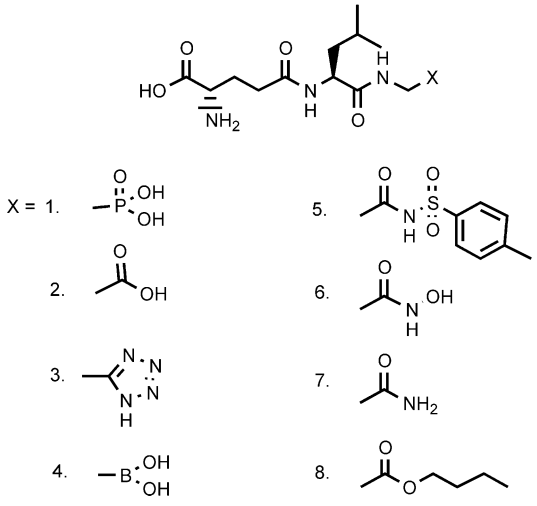


Figure 1. Analogues of glutathione (L-γ-Glu-L-Leu-Gly-X).

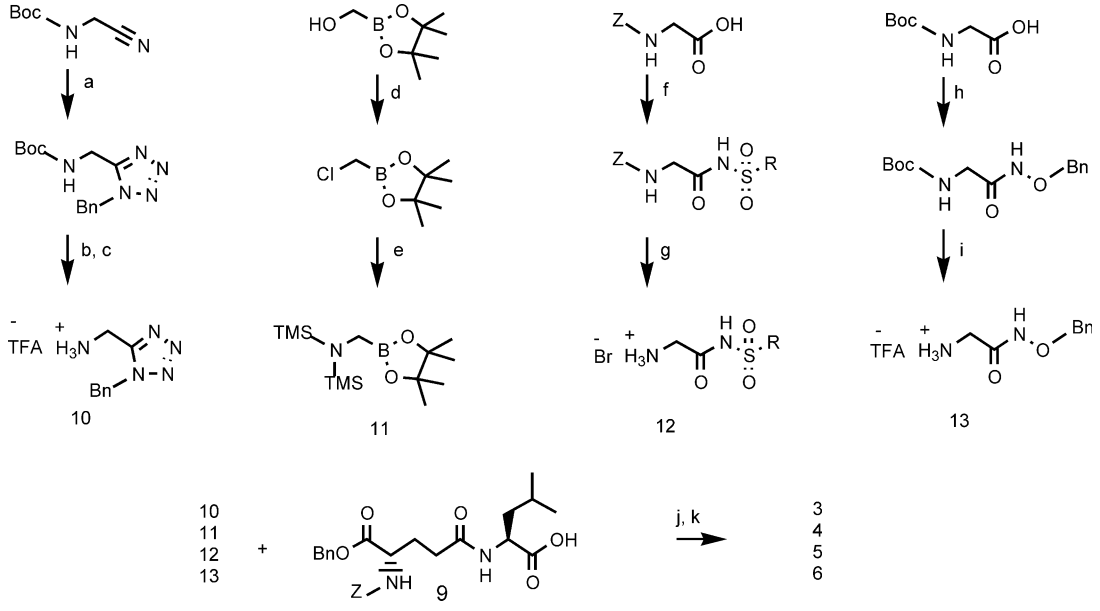
All pseudopeptides (Scheme 1) were prepared in a similar way. *N*-Z-L-γ-Glu(*O*Bn)-L-Leu (**9**) was prepared by coupling *N*-Z-L-Glu-*O*Bn and L-Leu-*Otert*-butyl with TBTU and deprotection with TFA.

Tripeptide **2** was synthesized as described.[9] Ester **8** was prepared from **2** by DCC/HONSu catalyzed esterification.

The tetrazole peptide **3** was prepared from 5-aminomethyl-1-benzyl-tetrazole (**10**), synthesized from *N*-Boc-protected aminoacetonitrile.[10] The carbonitrile function reacted with sodium azide to a tetrazole ring which was N^1-protected with a benzyl group. Coupling with protected L-γ-Glu-L-Leu and deprotection by hydrogenolysis afforded L-γ-Glu-L-Leu-5-aminomethyl-tetrazole (**3**).

The boronic acid peptide **4** was prepared from 2-bis (trimethylsilyl)aminomethyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**11**). The latter was prepared by amination of a chloroalkylboronate (prepared from 2-isopropyloxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane) with lithium hexamethyldisilazane[11] and coupled to protected L-γ-Glu-L-Leu (**9**) with TBTU. Deprotection by hydrogenolysis and acid work up afforded L-γ-Gly-L-Leu-aminomethylboronic acid HCl salt (**4**).

The peptide sulphonamide **5** was synthesized by the reaction of BOP-activated *Z*-glycine and the corresponding sulphonamide. Deprotection with HBr-HOAc afforded acylsulphonamide **12**, which was TBTU coupled to protected L-γ-Glu-L-Leu (**9**). Final deprotection with hydrogenolysis afforded L-γ-Glu-L-Leu-GlyNHSO$_2$ *p*-tolyl (**5**).



Scheme 1. Synthesis of L-γ-Glu-L-Leu-Gly-X peptides **3**–**6**: (a) NaN_3, NH_4Cl/DMF; (b) BnBr, Et_3N/CH_2Cl_2; (c) TFA/CH_2Cl_2; (d) $BrCH_2Cl$, BuLi/DMF; (e) lihexamethyldisilazane/THF; (f) BOP, NH_2SO_2R, Et_3N/DMF; (g) HBr/HOAc; (h) NH_2OBn, DCC, HOBt, Et_3N/DMF, CH_2Cl_2; (i) TFA; (j) TBTU, Et_3N/DMF or (**6**) DPPA, Et_3N/DMF; (k) H_2, Pd/C or (**3**) H_2, Pd/C, 5 h, 20 psi or (**5**) H_2, $Pd(OH)_2$/C (during the preparation of **4** the free boronic acid is formed during acid workup).

Table 1. Substrate and inhibitory activity of L-γ-Glu-L-Leu-$NHCH_2$-X against glutathionylspermidine synthetase (GspS) and the amidase free mutant

	X	Wild-type enzyme					C79A mutant				
		% substrate[a]	% inhibition[a]	IC_{50} (μM)	K_i (μM)	K_i' (μM)	% substrate[a]	% inhibition	IC_{50} (μM)	K_i (μM)	K_i' (μM)
1	$P{=}O(OH)_2$[b]	0	86	60±9	60±9						
2	COOH	104[c]	0	NA	NA	NA	125	0	NA	NA	
3	5-tetrazole	0	58	138±12							
4	$B(OH)_2$ HCl	1	95	17.2±0.8	81±35	18±2					
5	$CONHSO_2$*p*-Tol	2	2	2							
6	CONHOH	44	76				1	98	3.2±0.3	2.5±0.1	NA
7	$CONH_2$ TFA	93	0	0			22[d]	89[d]	105±9	NA	
8	$COOC_4H_9$	102	0				3	26			

[a]% activity compared to 0.5 mM GSH.
[b]Value taken from ref 6.
[c]Apparent K_m of 34±10 μM at concentrations <0.4 mM.
[d]This compound behaves as a competitive substrate with a K_m of 245±16 μM and a V_{max} that is 15% of that obtained with glutathione.
NA, not applicable.

The hydroxamic acid **6** was prepared from *N*-(*N*-Boc-glycyl)-*O*-benzylhydroxylamine (prepared with DCC/HOBt).[12] Boc-deprotection with TFA to *N*-glycyl-*O*-benzylhydroxylamine (**13**), DPPA coupling with protected L-γ-Glu-L-Leu (**9**) and hydrogenolysis afforded L-γ-Glu-L-Leu-GlyNHOH (**7**). The TBTU reaction between glycinamide and *N*-Z-L-γ-Glu(*O*Bn)-L-Leu (**9**) and deprotection furnished amide **7**.

All compounds[13] were tested as substrates and inhibitors of recombinant *C. fasciculata* GspS purified from *E. coli*[14] (Table 1). Tripeptide **2** behaved as a substrate under our assay conditions, whereas this had been found to be an inhibitor when tested at 5 mM under the conditions used by De Craecker et al.[9] A more detailed kinetic analysis revealed that compound **2** is indeed a substrate at low concentrations but showed pronounced substrate inhibition at higher concentrations, reconciling this apparent discrepancy. Tetrazole **3** was not active as a substrate but was a poor inhibitor with an IC_{50} of 138 μM. Boronic acid **4** behaved as a pure inhibitor, showing an IC_{50} of about 17 μM. *p*-Tolylsulphonamide **5** did not show any activity.

Hydroxamic acid **6** behaved as a substrate as well as an inhibitor of the wild-type enzyme. However, when assayed against a mutant form of GspS (C79A) that is devoid of amidase activity,[15] the compound no longer displayed activity as a substrate, but retained its potent inhibitory properties. HPLC analysis confirmed that the wild-type enzyme hydrolysed the hydroxamate moiety of **6** to produce the free carboxylate analogue **2**, which is a substrate rather than an inhibitor. The $t_{1/2}$ value of the hydrolysis by the amidase domain of the wild type enzyme was about 5.5 min with essentially complete hydrolysis after 1 h.

Amide **7** and butyl ester **8** were also completely hydrolysed by the amidase domain within 1 h to form **2** and therefore also behaved as substrates for the synthetase domain of GspS. A longer ester was however more slowly hydrolysed and showed weak activity as a substrate as well as being a weak inhibitor.

We conclude that substituting the carboxylic acid group in L-γ-Glu-L-Leu-Gly (**2**), a glutathione related substrate of GspS, affords inhibitory compounds in analogy to the already reported L-γ-Glu-L-Leu-Gly-phosphonic acid (**1**). The hydroxamate analogue **6** was the most potent competitive inhibitor of the synthetase (K_i 2.5 μM), but this was rapidly inactivated by hydrolysis by the amidase domain of the enzyme to form a substrate analogue L-γ-Glu-L-Leu-Gly. Since this peptide is itself a substrate for the synthetase, this results in a mixed substrate-inhibitor pattern. L-γ-Glu-L-Leu-Gly-boronic acid (**4**) appears the most promising inhibitor. It is not inactivated by the amidase domain and behaves as a mixed inhibitor of the synthetase (K_i' and K_i of 18 and 81 μM, respectively). Other substitutions behave as substrates, because they are hydrolysed by the amidase domain of GspS, or are not active.

Boronic acid (**4**) shows stronger inhibitory properties against GspS than the lead phosphonic acid compound (**1**).

Unfortunately, these compounds do not show any trypanocidal nor leishmanicidal activity in vitro.

Our results, however, provide interesting data for the further investigation of GspS as a target in anti-parasitic drug design. Efforts to improve the inhibitory activity of these compounds and to develop pro-drugs of these inhibitors are currently in progress.

Acknowledgements

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13. All compounds gave satisfactory spectroscopic data: We report the NMR, MS and HPLC data obtained with the most active compounds. HPLC on RP18 with A: H_2O–TFA (0.1%) and B: CH_3CN–TFA (0.1%) in a 35 min linear gradient (system I: from 10 to 90% B or system II: from 0 to 20% B).

(**3**) ^{1}H NMR (400 MHz) (CD_3OD) δ: 0.90 (d, 3H, $J=5.63$, δ-CH_3-Leu), 0.94 (d, 3H, $J=5.63$, δ-CH_3-Leu), 1.54–1.74 (m, 3H, β-CH_2- and γ-C*H*-Leu), 2.05–2.16 (m, 1H, β-CH_2-Glu), 2.17–2.28 (m, 1H, β-CH_2-Glu), 2.45–2.61 (m, 2H, γ-CH_2-Glu), 3.68–3.87 (m, 1H, α-C*H*-Glu), 4.31–4.45 (m, 1H, α-C*H*-Leu), 4.60–4.80 (2*H*, m, CH_2). ^{13}C NMR (CD_3OD) δ: 21.7 (δ-*C*H_3-Leu), 23.5 (δ-*C*H_3-Leu), 25.9 (γ-*C*H-Leu), 27.5 (β-*C*H_2-Glu), 32.5 (γ-*C*H_2-Glu), 41.5 (β-*C*H_2-Leu), 53.5 (α-*C*H-Leu), 54.9 (α-*C*H-Glu), 58.3 (CH_2), 130.0 (C), 175.0, 175.4 (CO). MS (FAB+) *m*/*z* 342 $[M+H]^+$. HPLC (A) R_t: 6.3 min; purity: 95%.

(**4**) ^{1}H NMR (400 MHz) (D_2O + two drops $(CD_3)_2CO$) δ: 0.80 (d, 3H, $J=5.36$, δ-CH_3-Leu), 0.84 (d, 3H, $J=5.98$, δ-CH_3-Leu), 1.42–1.69 (m, 3H, β-CH_2- and γ-C*H*-Leu), 1.92–2.18 (m, 2H, β-CH_2-Glu), 2.32–2.45 (m, 2H, γ-CH_2-Glu), 3.61–3.72 (m, 1H, α-C*H*-Glu), 4.25–4.35 (m, 1H, α-C*H*-Leu), 4.55–4.78 (m, 2H, *C*H_2). ^{13}C NMR (D_2O + two drops $(CD_3)_2CO$) δ: 20.9 (δ-*C*H_3-Leu), 22.4 (δ-*C*H_3-Leu), 24.5 (γ-*C*H-Leu), 26.4 (β-*C*H_2-Glu), 31.4 (γ-*C*H_2-Glu), 39.8 (β-*C*H_2-Leu), 51.4 (α-*C*H-Leu), 54.4 (α-*C*H-Glu), 63.0 (*C*H_2), 174.1, 175.3, 176.2 (*C*O). MS (ES+) *m*/*z* 317, 318 $[M+H]^+$. HPLC (B) R_t: 23 min; purity: >99%.

(**6**) ^{1}H NMR (400 MHz) (D_2O) δ: 1.03 (d, 3H, $J=5.9$, δ-CH_3-Leu), 1.08 (d, 3H, $J=5.6$, δ-CH_3-Leu), 1.70–1.90 (m, 3H, β-CH_2- and γ-C*H*-Leu), 2.20–2.40 (m, 2H, β-CH_2-Glu), 2.60–2.75 (m, 2H, γ-CH_2-Glu), 3.92 (m, 1H, α-C*H*-Glu), 4.04 (2*H*, m, CH_2), 4.48 (m, 1H, α-C*H*-Leu). MS (ES+) *m*/*z* 333 $[M+H]^+$. HPLC (B) R_t: 17.1 min; purity: >99%.

14. All compounds were initially tested as substrates and/or inhibitors at 0.5 mM using recombinant wild-type or C79A mutant *C. fasciculata* glutathionylspermidine synthetase-amidase. The relative activity as a substrate is the activity expressed as the % of activity measured with 0.5 mM glutathione. The % inhibition was determined according to the following equation:

$$\%\ \text{inhibition} = \left(1 - \frac{v_i}{v_o}\right).100$$

Initial velocity measurements were made at 8–10 inhibitor concentrations, ranging from 0 to 2.5 mM, and 0.5 mM glutathione. Spermidine (2 mM), Mg^{2+} (10 mM) and ATP (2 mM) were present in saturating conditions. The assays were conducted in 100 mM HEPES buffer pH 7.3 at 25 °C with 40 nM GspS. Assay mixtures were incubated for 5 min prior to initiation with spermidine. IC_{50} values were determined according to the following two parameter equation, where the lower data limit is 0, that is the data are background corrected, and the upper data limit is 100, that is the data are range corrected.

$$y = \frac{100}{1 + \left(\frac{x}{IC_{50}}\right)^s}$$

In this equation, *s* is a slope factor. The equation assumes that *y* falls with increasing *x*.

Initial velocity measurements were determined at three inhibitor concentrations and a serial dilution of glutathione, ranging from 0.03 to 1 mM. From these data, Michaelis–Menten and Lineweaver–Burk plots were constructed, and kinetic inhibitory values were determined by non-linear least squares regression analysis using the Grafit® programme. Inhibitors gave the best fit with mixed or competitive type inhibition. The indicated errors are the standard errors on the fit.

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