γ-L-Glutamyl-*O*-acyl-L-serylglycine Derivatives: Synthesis, Purification and Evaluation as Inhibitors of Glyoxalases

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Oxygen ester analogues of *S*-D-lactoylglutathione, γ-L-glutamyl-*O*-acyl-L-serylglycine derivatives, were synthesised and evaluated as inhibitors of glyoxalase II. They were competitive inhibitors where the inhibition constant K_i decreased with increase in acyl chain length. γ-L-Glutamyl-*O*-acyl-L-serylglycine derivatives also inhibit glyoxalase I. These compounds provide a novel route to glyoxalase II inhibitors for cancer chemotherapy.

The glyoxalase system catalyses the conversion of methylglyoxal to D-lactic acid, *via* the intermediate *S*-D-lactoylglutathione.[1] It comprises two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione. Glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from methylglyoxal and reduced glutathione.[2]

$$MeCOCHO + GSH \rightleftharpoons MeCOCH(OH)\text{–}SG \xrightarrow{\text{glyoxalase I}} MeCH(OH)CO\text{–}SG$$

Glyoxalase II (EC 3.1.2.6, hydroxyacylglutathione hydrolase) catalyses the hydrolysis of *S*-D-lactoylglutathione to D-lactate and regenerates the reduced glutathione consumed in the glyoxalase I-catalysed reaction.[3]

$$MeCH(OH)CO\text{–}SG + H_2O \xrightarrow{\text{glyoxalase II}} MeCH(OH)CO_2H + GSH$$

The glyoxalase system is present in the cytosol of cells and cellular organelles, particularly mitochondria.[1] It is found throughout biological life and is thought to be ubiquitous.[4] However, rapidly proliferating tumour cells have an unusual deficiency in glyoxalase II activity[5,6] and maintain a peculiarly high concentration of *S*-D-lactoylglutathione.[7] Addition of exogenous *S*-D-lactoylglutathione to human leukaemia cells in culture induces growth arrest and toxicity;[8] the low capacity of tumour cells to metabolise *S*-D-lactoylglutathione may give rise to its persistence and toxicity. Corresponding differentiated cells show no similar sensitivity to this and similar compounds.[9] Elevation of tumour cell concentrations of *S*-D-lactoylglutathione is, therefore, a prospective novel route to cancer chemotherapy. An efficient medicinal strategy to achieve this may be to inhibit the residual glyoxalase II activity in tumour cells (without significant inhibition of glyoxalase I activity).

S-Benzyloxycarbonylglutathione derivatives are strong competitive inhibitors of glyoxalase II. Inhibition constant K_i values for murine liver glyoxalase II were: *S*-benzyloxycarbonylglutathione $PhCH_2OCO\text{–}SG$ 65 μmol dm^{-3} and *p*-nitrobenzyloxycarbonylglutathione 6.5 μmol dm^{-3}.[10] Although these compounds were initially reported to be much less effective inhibitors of glyoxalase I than glyoxalase II,[10] data from subsequent investigations dispute this. The inhibitor constant K_i value for *S*-benzyloxycarbonylglutathiones for glyoxalase I (human red blood cell) was 1.26 μmol dm^{-3} and for glyoxalase II (bovine liver) was 13 μmol dm^{-3}.[11,12] More specific glyoxalase II inhibitors are required.

We chose to prepare oxygen ester analogues of the glyoxalase II substrate *S*-D-lactoylglutathione, *O*-acyl-seryl derivatives of γ-L-glutamyl-L-serylglycine (GOH). Oxygen esters are generally more stable to hydrolysis than thioester analogues and preservation of the γ-L-glutamyl-aminoacid-glycine backbone and ester carbonyl groups may provide improved specificity for potent inhibition of glyoxalase II and not glyoxalase I. We describe herein the synthesis and selected kinetic evaluation of GOH and its *O*-acetyl, propionyl, butyryl and valeryl esters.

Table 1 R_f values of synthetic intermediates

Compound	Solvent system[a] A	B	C	D
N-*t*-BOC-α-Benzylglutamate	0.49	0.78	0.78	0.81
4-Nitrophenol	0.58	0.98	0.93	0.90
N-*t*-BOC-α-Benzyl-γ-(4-nitrophenyl)-L-glutamate	1.00	1.00	1.00	1.00
N-*t*-BOC-α-Benzyl-γ-L-glutamyl-L-serylglycine	0.17	0.32	0.61	0.71
N-*t*-BOC-α-Benzyl-γ-L-glutamyl-L-seryl-benzylglycinate	0.85	ND[b]	ND	ND

[a] Solvent systems, chloroform–methanol–acetic acid (v/v/v): A, 90:8:2; B, 80:16:4; C, 70:24:6; D, 50:40:10. [b] ND = not determined.

Results and Discussion

Synthesis of N-t-*BOC-α-benzyl-γ-L-glutamyl-L-serylglycine.*—*N*-*t*-BOC-α-Benzyl-γ-L-glutamyl-L-serylglycine was synthesised by the coupling of *N*-*t*-BOC-α-benzyl-γ-(4-nitrophenyl)-L-glutamate with L-serylglycine. The 4-nitrophenyl ester was prepared as previously described by Chen *et al.*[13] with minor modification (3 recrystallisations were required) in good yield (66%) and purity. However, the method for the coupling reaction also described[13] could not be reproduced in the reaction time suggested (16 h). Rather, the reaction required 70–80 h to reach completion, as judged by the time course of formation of 4-nitrophenol, the cumulative addition of sodium hydroxide solution (to maintain the pH of the reaction mixture), and thin layer chromatography analysis (Table 1). The reason for the discrepancy is not clear.

The crystallised product form the coupling reaction contained impurities: 4-nitrophenol and *N*-*t*-BOC-α-benzyl-L-glutamic acid. Recrystallisation procedures failed to give a product of high purity. An inexpensive procedure for the purification of *N*-*t*-BOC-α-benzyl-γ-L-glutamyl-L-serylglycine with relatively

high capacity (1 g of crude product) was developed by using silica gel chromatography. The yield of pure product was 51% (based on L-serylglycine).

Table 2 Chromatographic R_f values and retention times t_r for γ-L-glutamyl-*O*-acyl-L-serylglycine derivatives (GO-R)

		Corrected retention time t_r'/min			
			preparative[c]		
R	R_f[a]	analytical[b]	A	B	C
H	0.42	0.3	0.3	0.5	0.8
Acetyl	0.51	3.2	ND[d]	ND	ND
Propionyl	0.57	4.9	1.1	ND	1.7
Butyryl	0.64	9.2	1.2	3.3	ND
Valeryl	0.70	12.9	2.3	ND	ND

[a] Thin layer chromatography: silica gel 60 plates with a mobile phase of propan-1-ol–acetic acid–water, 10:5:5; detection was by reactivity with ninhydrin (0.2% in ethanol) at 90 °C for 10 min. [b] Analytical reverse phase HPLC: column, 0.8 × 10 cm ODS radial compression cartridge with a 0.8 × 0.5 cm pre-column; mobile phase, 10 mmol dm^{-3} phosphoric acid, pH 2.83, with a 0–50% methanol gradient over 20 min; flow rate, 2 cm^3 min^{-1}. The dead time, t_0 was 1.5 min; detection was by absorbance flow spectrophotometry at 220 nm. [c] Preparative reverse phase HPLC: column, 2.5 × 10 cm ODS radial compression cartridge with a 2.5 × 1 cm pre-column; Flow rate 10 cm^3 min^{-1}. Mobile phase, 0.1% trifluoroacetic acid with: solvent system A, 0–80% acetonitrile over 10 min; system B, 0–20% acetonitrile over 10 min; and system C, isocratic without acetonitrile. Detection was by absorbance flow spectrophotometry at 220 nm. [d] ND = not determined.

Synthesis of γ-L-*glutamyl*-L-*serylglycine and* γ-L-*glutamyl*-O-*acyl*-L-*serylglycine Esters.*—γ-L-Glutamyl-L-serylglycine was prepared by catalytic hydrogenolysis and acid hydrolysis of the protecting groups of *N*-*t*-BOC-α-benzyl-GOH and purified by ion exchange chromatography on Dowex 1, formate form. γ-L-Glutamyl-*O*-acyl-L-serylglycine esters were prepared by benzylation of *N*-*t*-BOC-α-benzyl-γ-L-glutamyl-L-serylglycine by the caesium salt–benzyl bromide method [14] to *N*-*t*-BOC-α-benzyl-γ-L-glutamyl-L-seryl-benzyl-glycinate (protected tripeptide, 54% yield), acylation of the protected tripeptide with the appropriate acid anhydride and deprotection by catalytic hydrogenolysis–acid hydrolysis. The acyl-GOH derivative was purified by ion exchange chromatography on Dowex 1 (acetyl-GOH) or by preparative reverse phase HPLC (all other acyl-GOH derivatives) and analysed for purity by analytical reverse phase HPLC and silica gel TLC. TLC R_f values for synthetic intermediates are given in Tables 1. TLC R_f values and analytical and preparative HPLC retention times for GOH and acylated derivatives are given in Table 2. ^1H NMR spectra and assignments for acylated derivatives of GOH are given in Table 3.

Inhibition of Glyoxalases by γ-L-*Glutamyl*-O-*acyl*-L-*serylglycine Esters.*—The inhibition of bovine liver glyoxalase II was investigated for GOH, propionyl-GOH and valeryl-GOH, and inhibition of yeast glyoxalase I by GOH and valeryl-GOH. Kinetic data for the inhibition of glyoxalase II and glyoxalase I by valeryl-GOH are presented as Dixon plots in Fig. 1 and 2, and inhibition constant K_i values are given in Table 4. Incubation of GOH esters with glyoxalase II under assay conditions gave no GOH formation (by TLC analysis), confirming that these compounds are resistant to hydrolysis by glyoxalase II.

Table 3 Proton NMR spectrum[a] of γ-L-glutamyl-*O*-acyl-L- serylglycine derivatives

γ-L-Glutamyl-L-serylglycine derivative	GOH	*O*-Acetyl	*O*-Propionyl	*O*-Butyryl	*O*-Valeryl
Assignment					
Glycyl					
2-H (2 H)	3.89	3.88	3.89	3.91	3.90
Seryl					
2-H	4.40	4.62	4.61	4.62	4.62
3A-H	3.79	4.38	4.37	4.38	4.36
3B-H	3.77	4.24	4.24	4.25	4.23
$J_{2,3A}$	5.16	5.16	5.55	5.56	5.56
$J_{2,3B}$	5.16	4.17	4.37	4.55	4.37
$J_{3A,3B}$	—	−11.61	−11.51	−11.61	−11.51
γ-L-Glutamyl					
2-H	3.77	3.73	3.86	3.88	3.93
3-H (2 H)	2.08	2.06	2.09	2.10	2.11
4-H (2 H)	2.47	2.45	2.46	2.48	2.48
$J_{2,3}$	7.94	6.35	6.55	6.55	6.55
$J_{3,4}$	6.75	8.01	7.94	7.47	7.54
Other		Acetyl	Propionyl	Butyryl	Valeryl
		2-H (3 H)	2-H (2-H)	2-H (2 H)	2-H (2 H)
		1.99	2.29	2.10	2.28
			3-H (3 H)	3-H (2 H)	3-H (2 H)
			0.96	1.49	1.43
				4-H (3 H)	4-H (2 H)
				0.79	1.17
					5-H (3 H)
			$J_{2,3}$	$J_{2,3}$	$J_{2,3}$
			7.54	7.54	7.54
				$J_{3,4}$	$J_{3,4}$
				7.34	7.94
					$J_{4,5}$
					7.30

[a] Recorded at 270 MHz in D_2O. Chemical shifts δ are in ppm and coupling constants *J* in Hz.

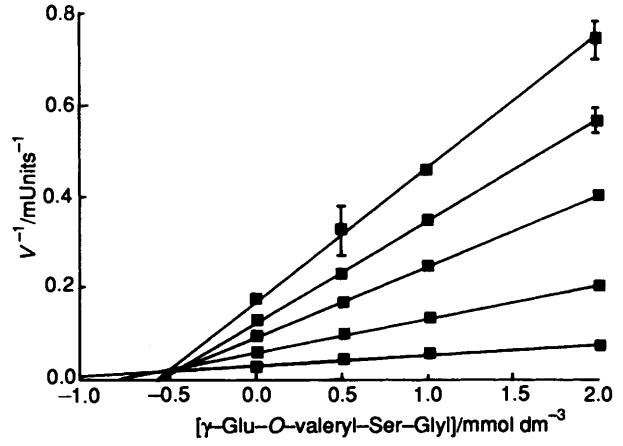

Fig. 1 Dixon plot for the inhibition of glyoxalase II by γ-L-glutamyl-*O*-valeryl-L-seryglycine. Data are the mean ± standard deviation for three determinations. The K_M value (mean ± standard deviation) was 214 ± 15 μmol dm^{-3} and the apparent v_{max} was 56.5 ± 2.8 mUnits cm^{-3} (Eadie–Hofstee).

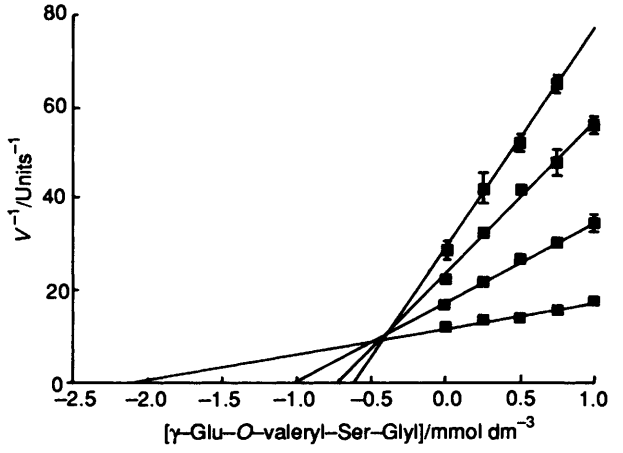

Fig. 2 Dixon plot for the inhibition of glyoxalase I by γ-L-glutamyl-*O*-valeryl-L-serylglycine. Data are the mean ± standard deviation for three determinations. The K_M value (mean ± standard deviation) was 102 ± 7 μmol dm^{-3} and the apparent v_{max} was 94.6 ± 4.9 mUnits cm^{-3} (Eadie–Hofstee).

Table 4 Inhibition constants for the competitive inhibition of glyoxalases by γ-L-glutamyl-*O*-acyl-L-serylglycine derivatives[a]

Inhibitor GO-R R	K_i value/mol dm^{-3}	
	Glyoxalase I	Glyoxalase II
H	7.37 ± 0.56	5.32 ± 0.19
Propionyl	ND[b]	1.55 ± 0.05
Valeryl	0.412 ± 0.048	0.488 ± 0.080

[a] Data are the mean ± standard deviation of the abscissa coordinate of the intercepts of linear regression lines of experimental data (n = 10). [b] ND = not determined.

The inhibition of glyoxalase II by GOH derivatives was competitive with K_i values decreasing with increasing carbon chain length on the acyl group. However, GOH derivatives also inhibit glyoxalase I to give similar K_i values; they did not, however, inhibit the formation of the hemithioacetal substrate. There is significant variation of K_i values for glyoxalases from different sources,[11] therefore, no deduction about the possible inhibition of human enzymes by these compounds may be made from these data.

More potent glyoxalase II inhibitors may be produced from GOH esters with greater hydrophobic characteristics. Also, kinetic data of more immediate medicinal interest may be gained from studying the inhibition of human glyoxalases by GOH derivatives. This work describes working methods to prepare novel GOH derivatives which may lead to the development of compounds suitable for further biological evaluation.

Experimental

Materials.—L-Serylglycine and *N-t*-BOC-α-benzyl-L-glutamic acid were purchased from Bachem Chemical Company (Bubendorf, Switzerland). 4-Nitrophenol, dicyclohexylcarbodiimide, acetic anhydride, propionic anhydride, butyric anhydride, valeric anhydride and benzyl bromide were obtained from Aldrich Chem. Co. (Dorset, UK). Glyoxalase I from yeast and glyoxalase II from bovine liver, Celite and Dowex 1 anion exchange resin were purchased from Sigma Chem. Co. (Poole, Dorset, UK). *S*-D-Lactoylglutathione was synthesised and purified as previously described.[15] Pyridine was refluxed with potassium hydroxide pellets, distilled (b.p. 113–114 °C) and stored over potassium hydroxide pellets. Silica gel 60, 230–400 mesh, and silica gel 60 F_{254} TLC plates were purchased from Whatman (New Jersey, USA). Biogel P-2 matrix was purchased from Biorad (Abingdon, Oxfordshire, U.K.).

Instrumentation.—High performance liquid chromatography (HPLC) was performed with a Waters HPLC system (2 × 510 pumps, Lambda-Max 481 LC spectrophotometer with a 680 automated gradient controller). Reverse phase, radial compression cartidges were used in a RCM-100 (analytical) and a RCM 25 × 10 (preparative) radial compression modules. NMR spectra were recorded at 270 MHz on a JEOL EX 270 NMR spectrometer; chemical shifts are reported as δ-values in ppm and coupling constants (J) are in Hz. Specific rotations are measured in 10^{-1} deg cm^2 g^{-1}.

N-t-*BOC-α-Benzyl-γ-(4-nitrophenyl)-L-glutamate.*—This was prepared from *N-t*-BOC-L-glutamic acid α-benzyl ester as described by Chen *et al.*[13] except that the product was recrystallised three times from absolute ethanol to give white asters of crystals of *N-t*-BOC-α-Benzyl-γ-(4-nitropheny)-L-glutamate, $\delta_H(CD_3CN)$, 1.51 (s, 9 H, BOC *tert*-butyl), 2.10 (unres., 2 H, β-CH_2), 2.81 (t, 2 H, J 7.3, γ-CH_2 Glu), 4.40 (unres., 1 H, α-CH_2), 5.27 (s, 2 H, benzyl CH_2), 7.44 (d, 2 H, $J_{o,m}$ 9.1, 4-nitrophenyl *ortho*-aromatic), 7.49 (s, 5 H, α-benzyl aromatic) and 8.37 (d, 2 H, $J_{o,m}$ 8.50, 4-nitrophenyl *m*-aromatic); m.p. 98.100 °C; $[\alpha]_D^{20}$ −16.3 (ethylacetate); yield 66%.

N-t-*BOC-α-Benzyl-γ-L-glutamyl-L-serylglycine.*—The coupling of *N-t*-BOC-α-Benzyl-γ-(4-nitrophenyl)-L-glutamate active ester to L-serylglycine was accomplished in aqueous active pyridine by modification of the method by Chen *et al.*[13] L-Serylglycine (1.00 g, 6.18 mmol) was dissolved in pyridine (8 cm^3) and water (4 cm^3), the pH was adjusted to 8.77 by aqueous NaOH (5 mol dm^{-3}, 1.06 cm^3) at 25 °C. Active ester (0.82 g, 1.85 mmol) and pyridine (2 cm^3) were then added. A further three equal additions were made at 4 h intervals such that a total of 3.28 g (7.40 mmol) of active ester was added. The pH was maintained at 8.5–8.7 by further addition of aqueous NaOH (5 mol dm^{-3}). During method development, an aliquot of the reaction mixture (10 mm^3) was taken at time points during the reaction and analysed spectrophotometrically at 438 nm. The concentration of 4-nitrophenol was deduced from a calibration curve of standard solutions. The cumulative addition of NaOH solution, the formation of 4-nitrophenol and TLC analysis were used to follow the progress of the reaction (Table 1).

When the reaction had reached completion (*ca.* 70–80 h), $NaHCO_3$ (6.0 g in 50 cm^3 of water) was added and the mixture extracted with ethyl acetate (2 × 20 cm^3). The organic layer was back extracted twice with water (10 cm^3). The aqueous phase and extracts were combined and residual ethyl acetate was removed under reduced pressure. The pH of the solution was adjusted to 2.47 with conc. HCl and left to crystallise at 4 °C. After 4 d, the white solid was collected by filtration to give 2.15 g of crude product.

Crude product (1.00 g) was dissolved in chloroform–methanol–acetic acid, 90:8:2, and applied to a silica gel column (2.5 × 23 cm) equilibrated with the same solvent. The column was eluted with mobile phase with a flow rate of 75 cm^3 h^{-1} and 5 cm^3 fractions were collected. The fractions obtained were analysed spectrophotometrically at 275 nm and by TLC (silica gel with the same mobile phase developing solvent). Fractions 14–20 contained 4-nitrophenol (R_f 0.58) and *N-t*-BOC-α-benzyl-γ-L-glutamate (R_f 0.49), and fractions 23–30, the protected tripeptide (R_f 0.17). Fractions 23–30 were pooled and evaporated to dryness under reduced pressure. The oily residue was dissolved in 50% aqueous ethanol (10 cm^3) and lyophilised to give 0.71 g (1.47 mmol) of white solid product. *N-t*-BOC-α-Benzyl-γ-L-glutamyl-L-serylglycine: $\delta_H(CD_3CN)$ 1.40 (s, 9 H, BOC *tert*-butyl), 1.96 (unres., 2 H, β-CH_2 Glu), 2.35 (t, 2 H, *J* 6.95, γ-CH_2 Glu), 3.68 (q, 1 H, $J_{2.3A}$ 4.8, $J_{3A.3B}$ −11.5, 3 HA-Ser), 3.79 (q, 1 H, $J_{2.3B}$ 5.0, $J_{3A.3B}$ −11.5, 3 HB-Ser), 3.9 (d, 2 H, $J_{2H.NH}$ 6.0, CH_2 Gly), 4.17 (unres., 1 H, α-CH Glu), 4.38 (unres., 1 H, α-CH Ser), 5.15 (s, 2 H, CH_2 benzyl) and 7.38 (s, 5, H, benzyl); one peak by analysis on reverse phase HPLC, retention time 11 min (column, 0.8 × 10 cm ODS radial compression cartridge with a 0.8 × 0.5 cm pre-column; mobile phase, 0.1% trifluoroacetic acid with 50% acetonitrile; flow rate, 2 cm^3 min^{-1}).

γ-L-*glutamyl*-L-*serylglycine* (*GOH*).—*N-t*-BOC-α-Benzyl-γ-L-glutamyl-L-serylglycine (0.62 g, 1.27 mmol) was dissolved in trifluoroacetic acid (5 cm^3) and stirred with palladium on active carbon (10%, 40 mg) under hydrogen gas (1 atm) at room temperature for 6 h. Distilled water (2 cm^3) was added to the reaction mixture and it was then filtered through Celite. The filtrate was lyophilised, reconstituted with hydrochloric acid (1 mol dm^3, 10 cm^3) and lyophilised twice, and the residual solid was purified by column chromatography on Dowex 1, formate form (column, 2.6 × 34 cm; mobile phase, 20 mol dm^{-3} formic acid 100 cm^3, followed by a gradient of 20–500 mmol dm^{-3} formic acid, 1200 cm^3; flow rate 70 cm^3 h^{-1}; fraction size, 12.5 cm^3). Fractions were analysed by TLC (silica gel, propan-1-ol–acetic acid–water, 10:5:5, detection by ninhydrin), those containing a spot of R_f 0.45 were pooled and lyophilised to give the product (0.21 g, 0.71 mmol, 56%). γ-L-Glutamyl-L-serylglycine was characterised by ^1H NMR. (Table 3) and gave one spot by silica gel TLC analysis and one peak in analysis by analytical reverse phase HPLC (Table 2).

N-t-*BOC*-α-*Benzyl*-γ-L-*glutamyl*-L-*seryl-benzylglycinate*.—*N-t*-BOC-α-Benzyl-γ-L-glutamyl-L-serylglycine (1.00 g, 2.08 mmol) was dissolved in 90% methanol–water (50 cm^3) and the pH adjusted to 7.0 by addition of caesium carbonate solution (20% in water). The solution was evaporated under reduced pressure, reconstituted with 50 cm^3 of absolute ethanol and evaporated under reduced pressure twice, and the residual solid dissolved in dimethylformamide (50 cm^3). Benzyl bromide (0.23 cm^3) was added and the solution stirred at 40 °C for 48 h. Distilled water (50 cm^3) was then added and the reaction mixture was extracted with ethyl acetate (2 × 40 cm^3). The organic layer was washed twice with water (40 cm^3), dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to dryness. The residual solid was dissolved in chloroform–methanol–acetic acid, 90:8:2, and loaded onto a column of silica gel (2.5 × 21 cm) equilibrated with the same solvent. The column was eluted with the same mobile phase; 5 cm^3 fractions were collected and analysed by TLC with the same mobile phase. Those containing one spot with R_f 0.85 (Table 1) were pooled and evaporated under reduced pressure to dryness to give a white solid, *N-t*-BOC-α-benzyl-γ-L-glutamyl-L-seryl-benzylglycinate, $\delta_H(CD_3CN)$ 1.40 (s, 9 H, BOC *tert*-butyl), 2.00 (unres., 2 H, β-CH, Glu), 2.33 (unres., 2 H, γ-CH_2 Glu), 3.95 (d, 2 H, $J_{2\text{-}H.NH}$ 6.0, β-CH_2 Gly), 4.14 (unres., 2 H, β-CH_2 Ser), 4.36 (unres., 1 H, α-CH Glu), 4.57 (unres., 1 H, α-CH Ser), 5.12 and 5.14 (s, 2 H each, α-benzyl Glu and α-benzyl Gly CH_2) and 7.37 (unres., 10 H, α-benzyl Glu and α-benzyl Gly aromatic); one spot by TLC analysis (Table 1).

γ-L-*Glutamyl*-O-*acetyl*-L-*serylglycine* [*Acetyl-GOH*].—*N-t*-BOC-α-Benzyl-γ-L-glutamyl-L-seryl-benzylglycinate (0.50 g, 0.88 mmol) was dissolved in acetonitrile (6 cm^3). Acetic anhydride (0.17 cm^3) and pyridine (71.4 mm^3) were added and the reaction mixture was stirred for 24 h at room temperature. The solvent was then removed under reduced pressure. The crude product was recrystallised from ethanol–water to give 0.412 g of acetylated intermediate. The solid was dissolved in methanol (5 cm^3) and stirred with palladium on carbon (10%, 41 mg) under hydrogen gas (1 atm) for 4 h. Distilled water (1 cm^3) was added and the reaction mixture was filtered through Celite and evaporated to dryness under reduced pressure. The residual solid was dissolved in trifluoroacetic acid (5 cm^3), stirred for 60 min and evaporated to dryness to give 0.310 g of crude product. This crude material was dissolved in distilled water (1 cm^3) and applied to a Bio-gel P-2 column (2.6 × 40 cm). The column was eluted with distilled water and fractions (2 cm^3) were collected. The fractions were analysed by TLC (silica gel, mobile phase propan-1-ol–acetic acid–water, 10:5:5; detection with ninhydrin) and samples with one spot of R_f 0.48 were pooled and lyophilised to give 12 mg (4%) of product. This was identified as Acetyl-GOH by ^1H NMR (Table 3).

γ-L-*glutamyl*-O-*propionyl*-L-*serylglycine* (*Propionyl-GOH*), γ-L-*glutamyl*-O-*butyryl*-L-*serylglycine* (*Butyryl-GOH*) *and* γ-L-*Glutamyl*-O-*valeryl*-L-*serylglycine* (*Valeryl-GOH*).—The procedure used was similar to the above except for using the appropriate acid anhydride as the acylating agent. The product was purified by preparative reverse phase HPLC (Table 2). The desired product fraction was collected and lyophilised to give 49 mg (16%) of propionyl-GOH, 49 mg (16%) of butyryl-GOH and 167 mg (51%) of valeryl-GOH. Acylated GOH derivatives were characterised by ^1H NMR (Table 3) and the purity assessed by TLC and analytical HPLC (Table 2).

Kinetic Studies of Glyoxalase I and Glyoxalase II.—*Glyoxalase I.* The rate of the glyoxalase I-catalysed reaction was followed by measuring the initial rate of formation of *S*-D-lactoylglutathione from hemithioacetal formed non-enzymically from methylglyoxal and reduced glutathione, followed spectrophotometrically at 240 nm; Δε 2.86 $mmol^{-1}$ dm^3 cm^{-1}.[5] Methylglyoxal and reduced glutathione were preincubated for 10 min in 50 mol dm^{-3} sodium phosphate buffer, pH 6.6 and 37 °C, to form the hemithioacetal substrate. Glyoxalase I (0.05 U cm^{-3}) and inhibitor were then added. The effect of the inhibitor on the formation of hemithioacetal was followed by studying the change in absorbance at 240 nm during the 10 min pre-incubation. The nominal hemithioacetal concentrations used were 627, 170, 87.5 and 63.5 μmol dm^{-3}. One unit of glyoxalase I activity represents the formation of 1 μmol of *S*-D-lactoylglutathione per minute under assay conditions.

Glyoxalase II. The rate of the glyoxalase II-catalysed reaction was followed by measuring the initial rate of hydrolysis of

synthetic *S*-D-lactoylglutathione followed spectrophotometrically at 240 nm; $\Delta\varepsilon$ -3.1 mmol^{-1} dm^3 cm^{-1}.[5] *S*-D-Lactoylglutathione, glyoxalase II (0.02 U cm^{-3}) and inhibitor were incubated in 50 mmol dm^{-3} TRIS·HCl buffer, pH 7.4 and 37 °C. One unit of glyoxalase II activity represents the hydrolysis of 1 μmol of *S*-D-lactoylglutathione per minute under assay conditions. The initial *S*-D-lactoylglutathione concentrations were 333, 100, 50, 33 and 25 μmol dm^{-3}.

Data are the mean ± standard deviation of three determinations and were manipulated for the Dixon plot with least squares linear regression of the experimental data. The inhibitor constants quoted are the mean ± standard deviation of the ordinate values from intercepts of the least squares regression.

Acknowledgements

We thank the Leukaemia Research Fund for support for our research programme. This is a contribution from the Glyoxalase Research Group at the University of Essex.

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Paper 1/04766C
Received 13*th September* 1991
Accepted 1*st November* 1991