S-2-Hydroxyacylglutathione-Derivatives: Enzymatic Preparation, Purification and Characterisation

James D. Clelland and Paul J. Thornalley
Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

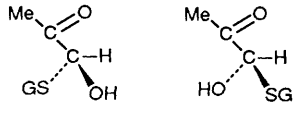
S-2-Hydroxyacylglutathione derivatives have been prepared by enzymatic synthesis from α-oxo aldehydes and reduced glutathione in the presence of glyoxalase I. *S*-D-Lactoylglutathione, *S*-D-mandelylglutathione, *S*-glycolylglutathione and *S*-L-glyceroylglutathione were prepared from methylglyoxal, phenylglyoxal, glyoxal and hydroxypyruvaldehyde, respectively. They were purified by ion exchange chromatography on Dowex 1 on a gram scale. Analytical data and re-evaluated extinction coefficients for these compounds are presented.

The method described provides a reliable, large-scale procedure for the preparation and purification of *S*-acylglutathiones of increasing biological and pharmacological interest.

S-2-Hydroxyacylglutathione derivatives are formed from α-oxo aldehydes and reduced glutathione by the enzymatic reaction of glyoxalase I (EC 4.4.1.5). α-Oxo aldehyde and reduced glutathione react non-enzymatically to form the hemithioacetal adduct. Glyoxalase I catalyses the isomerisation of hemithioacetal to *S*-2-hydroxyacylglutathione.[1] Glyoxalase I accepts a

$$\mathrm{RCOCHO} + \mathrm{GSH} \rightleftharpoons \mathrm{RCOCH(OH)\text{-}SG} \xrightarrow{\text{Glyoxalase I}} \mathrm{RCH(OH)CO\text{-}SG}$$

broad range of α-oxo aldehydes for this reaction[2,3] although the physiological substrate of the enzyme is thought to be the hemithioacetal derived from methylglyoxal producing *S*-D-lactoylglutathione.[1] The reaction is stereospecific, producing the D-lactoyl stereoisomer.[4] The reaction mechanism is thought to involve the formation of an enzyme bound enediolate intermediate.[5,6] The hemithioacetal is the molecular species which binds to the enzyme active site; both (*S*)- and (*R*)-forms



(*S*)-Substrate (*R*)-Substrate

of the hemithioacetal are formed non-enzymatically from α-oxo aldehyde and reduced glutathione in solution. The glyoxalase I-catalysed isomerisation of the hemithioacetal to *S*-D-hydroxyacylglutathione proceeds through a *cis*-enediolate intermediate, formed from the (*S*)-hemithioacetal.[7] However, both (*S*)- and (*R*)-forms appear to be consumed[8-10] which may be due to enzyme-catalysed epimerisation of the (*R*)- to the (*S*)-hemithioacetal[7] or rapid equilibrium between (*R*)- and (*S*)-forms.

Recent interest in the biological and pharmacological properties of *S*-D-lactoylglutathione and other *S*-2-hydroxyacylglutathione derivatives has been raised by the discovery that *S*-D-lactoylglutathione induces growth arrest and toxicity in human leukaemia cells in culture.[11] Further biological and medicinal evaluation of this effect requires the large scale synthesis and purification of *S*-2-hydroxyacylglutathiones to high purity.

Here, we provide a detailed description of the large-scale enzymatic synthesis and purification of *S*-2-hydroxyacylglutathione derivatives. Analytical data and re-evaluated extinction coefficients are also presented.

Results and Discussion

Enzymatic Synthesis of S-2-*Hydroxyacylglutathione Derivatives from Reduced Glutathione and* α-*Oxo Aldehydes.*—When reduced glutathione (10 mmol dm^{-3}) was incubated with α-oxo aldehyde (10 mmol dm^{-3}) in sodium phosphate buffer (5 mmol dm^{-3}; pH 6.6), in the presence of glyoxalase I (90 units), *S*-2-hydroxyacylglutathione derivatives were rapidly formed (Figs. 1–4, part *a*). With commercial yeast glyoxalase I, higher initial concentrations of α-oxo aldehyde inhibited the reaction progress (data not shown). For methylglyoxal [Fig. 1(*a*)], there was rapid formation of *S*-lactoylglutathione in the initial 20 min of incubation, which reached a maximum after 30 min and slowly decreased thereafter. Similar incubations with glyoxal [Fig. 2(*a*)], hydroxypyruvaldehyde [Fig. 3(*a*)] and phenylglyoxal [Fig. 4(*a*)] required longer incubation times to reach a maximum for conversion of each into *S*-glycolylglutathione, *S*-glyceroylglutathione and *S*-mandelylglutathione, respectively; these increases in time reflected the relative reactivity of these substrates with yeast glyoxalase I.[2] The initial concentration of α-oxo aldehyde was 10 mmol dm^{-3}. At concentrations greater than this, the progress of reaction was impeded by inhibition of the enzyme. Yeast (and other microbial procaryotic forms of glyoxalase I) are particularly sensitive to inhibition by high concentrations of α-oxo aldehyde since there is an arginyl residue in the active site;[12] mammalian forms of glyoxalase I do not have this and are resistant to inhibition by high α-oxo aldehyde concentrations. (We persisted with the yeast enzyme in these experiments because of its commercial availability and therefore the method developed would be generally accessible to non-specialist research groups.)

Purification of S-2-*Hydroxyacylglutathione Derivatives by Ion Exchange Chromatography on Dowex* 1.—When samples of *S*-2-hydroxyacylglutathiones synthesised from 3 mmol of glutathione–methylglyoxal reaction mixture, free of glyoxalase I after ultrafiltration, were loaded onto a column of Dowex 1, formate form and eluted with 20–620 mmol dm^{-3} formic acid, separation from trace contaminants was achieved (Figs. 1–4, part *b*). Samples were treated with diamide, oxidised form, to convert reduced glutathione into oxidised glutathione to improve separation. Oxidised diamide [1,1′-azo(*N*,*N*-dimethylformamide)] and reduced diamide [1,1′-hydrazo(*N*,*N*-dimethylformamide)] were eluted first—peaks I and II. For *S*-lactoylglutathione, *S*-glycolylglutathione and *S*-glyceroylglutathione, peak III was the *S*-2-hydroxyacylglutathione, and peak IV was oxidised glutathione [Figs. 1(*a*), 2(*b*) and 3(*b*)]. For *S*-mandelylglutathione, oxidised glutathione (peak III)

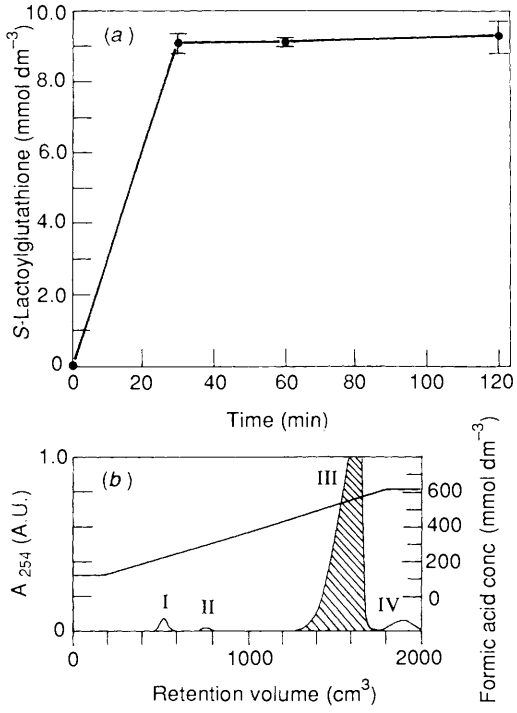


Fig. 1 Synthesis and purification of *S*-lactoylglutathione. (*a*) Enzymatic formation. Incubation mixtures contained 10 mmol dm^{-3} methylglyoxal, 10 mmol dm^{-3} reduced glutathione and glyoxalase I (90 units) in 5 mmol dm^{-3} sodium phosphate buffer, pH 6.6 and 37 °C. The concentration of *S*-lactoylglutathione was assayed spectrophotometrically in the presence of glyoxalase II (see Materials and Methods section). Data are the mean ± standard deviation of 4 determinations. (*b*) Chromatographic purification. Crude samples (3 mmol, 10 ml) of *S*-lactoylglutathione were dissolved in initial mobile phase, titrated with diamide and adjusted to the pH of the column. The column (5 × 30 cm) was eluted with formic acid using the concentration profile indicated: flow rate 300 cm^3 h^{-1}, fraction size 15 cm^3. The eluate was monitored at 254 nm (see Table 5 for extinction coefficients).

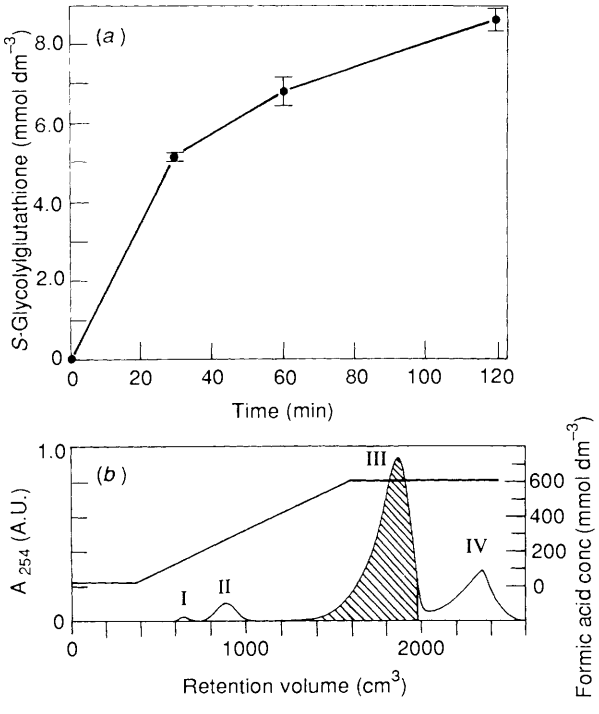


Fig. 2 Synthesis and purification of *S*-glycolylglutathione. (*a*) Enzymatic synthesis; (*b*) chromatographic purification. Details as for Fig. 1.

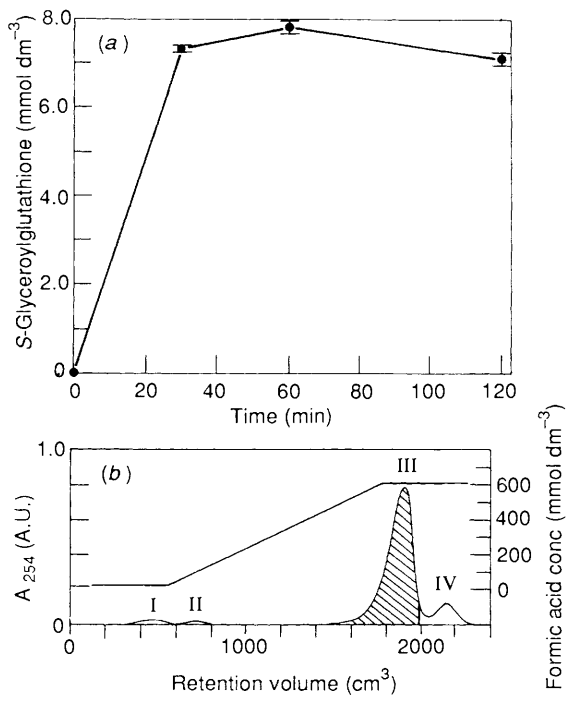


Fig. 3 Synthesis and purification of *S*-glyceroylglutathione. (*a*) Enzymatic syntheses. (*b*) Chromatographic purification. Details as for Fig. 1.

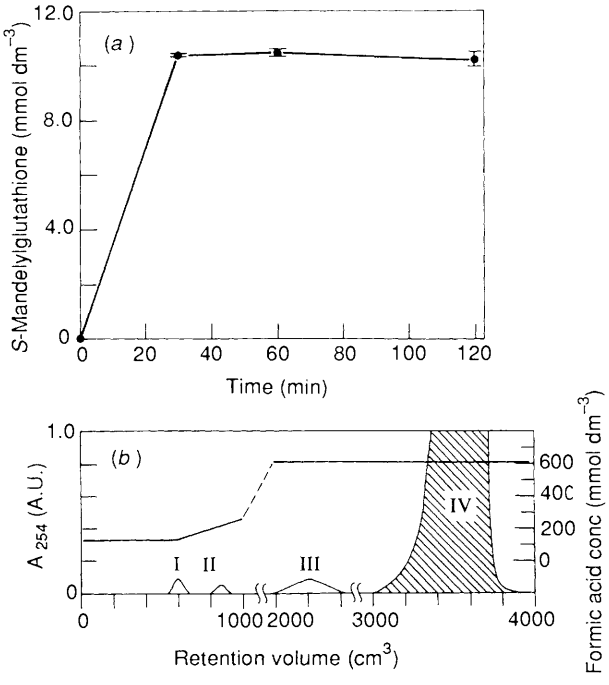


Fig. 4 Synthesis and purification of *S*-mandelylglutathione. (*a*) Enzymatic synthesis. (*b*) Chromatographic purification. Details as for Fig. 1.

was eluted before *S*-mandelylglutathione (peak IV) [Fig. 4(*b*)].

Purification of the synthesised compounds by ion exchange chromatography provided a product of high purity with gram scale sample loading. Diamide was added to the synthesised product immediately prior to chromatography in formic acid solution to oxidise reduced glutathione to oxidised glutathione. *S*-Lactoylglutathione, *S*-glycolylglutathione and *S*-glyceroylglutathione were resolved from oxidised glutathione (Fig. 2) but not from reduced glutathione on Dowex 1. Diamide reacted

Table 1 Proton chemical shifts and spin-spin coupling constants for *S*-2-hydroxyacylglutathione derivatives in 100 mmol dm^{-3} NaD_2PO_4/Na_2DPO_4, pD 7.0

Compound	Reduced glutathione	*S*-Glycolyl-glutathione	*S*-Lactoyl-glutathione	*S*-Glyceroyl-glutathione	*S*-Mandelyl-glutathione
Assignment δ (*J*)					
Glycyl					
1A-H	3.62	3.68	3.68	3.68	3.60
1B-H	3.62	3.61	3.60	3.60	3.44
($J_{1A,1B}$	—	−17.1	−17.5	−17.5	−17.1)
Cysteinyl					
2-H	4.42	*	4.31	*	4.61
3A-H	2.83	3.38	3.35	3.35	3.35
3B-H	2.77	3.12	3.07	3.11	3.08
($J_{2,3A}$	5.4	4.8	4.8	4.8	5.2)
($J_{2,3B}$	6.7	8.1	8.3	8.2	8.3)
($J_{3A,3B}$	−14.3	−14.3	−14.3	−14.3	−14.3)
γ-Glutamyl					
2-H	3.63	3.68	3.64	3.64	3.59
3-H (2 H)	2.01	2.02	2.02	2.01	1.93
4-H (2 H)	2.40	2.37	2.37	2.37	2.26(A) 2.23(B)†
($J_{2,3}$	7.1	6.8	6.7	7.1	7.2†)
($J_{3,4}$	7.1	7.9	7.2	7.1	6.4†)
Other					
		Glycolyl	Lactoyl	Glyceroyl	Mandelyl
		2-H (2 H) = 4.20	2-H = 4.28 3-H (3 H) = 1.25 ($J_{2,3}$ = 6.8)	2-H = 4.28 3-H (2 H) = 3.70 ($J_{2,3}$ = 4.4)	2-H = 5.26 Ph = 7.34

Chemical shifts δ_H(ppm) and coupling constants J_{H_x,H_y}/Hz for spectra recorded at 270 MHz for 10 mg ml^{-1} solutions in 100 mmol dm^{-3} NaD_2PO_4/Na_2DPO_4, pD 7.0. * 2-H of Cys absent due to suppression of HOD resonance. † 8 Hyperfine components partially resolved indicating inequivalence of all 3- and 4-protons.

Table 2 ^{13}C NMR spectroscopy of *S*-2-hydroxyacylglutathiones in 100 mmol dm^{-3} sodium phosphate buffer, pH 6.6

Compound	Reduced glutathione	*S*-Glycolyl-glutathione	*S*-Lactoyl-glutathione	*S*-Glyceroyl-glutathione	*S*-Mandelyl-glutathione
Assignment Glycyl					
C-1	174.5	174.4	174.5	175.2	174.9
C-2	42.0	41.8	42.1	42.5	42.4
Cysteinyl					
C-1	171.6	171.3	171.1	171.0	170.8
C-2	55.2	53.5	53.6	53.7	53.7
C-3	25.0	25.6	25.7	25.7	25.7
γ-Glutamyl					
C-1	173.4	173.3	173.4	173.4	173.4
C-2	53.6	52.3	52.2	52.2	52.1
C-3	25.6	28.3	28.6	28.7	28.7
C-4	30.7	30.7	30.8	30.8	30.8
C-5	174.5	174.3	174.3	174.3	174.1
Other					
		Glycolyl	Lactoyl	Glyceroyl	Mandelyl
		C-1 204.0 C-2 66.7	C-1 207.0 C-2 73.0 C-3 19.2	C-1 204.4 C-2 77.4 C-3 62.8	C-1 204.3 C-2 78.7 C-1(Ph) 137.1 C-2,6(Ph) 128.6 C-3,5(Ph) 126.4 C-4(Ph) 128.7

Table 3 FABS mass spectrometric analysis of *S*-2-hydroxyacylglutathiones

Compound	$(M + H)^+$
S-Glycolylglutathione	366
S-Lactoylglutathione	380
S-Glyceroylglutathione	396
S-Mandelylglutathione	442

with reduced glutathione, forming oxidised glutathione and reduced diamide.

$$Me_2NCO\text{–}N{=}N\text{–}CONMe_2 + 2GSH \longrightarrow Me_2NCONHNHCONMe_2 + GSSG$$

There was no reaction with *S*-2-hydroxyacylglutathione.[5] However, our recent purification of radiolabelled *S*-D-lactoylglutathione, containing some residual [2-^{14}C]methylglyoxal, by this method suggests both oxidised and reduced diamide may bind α-oxo aldehyde.[13] This procedure provides good chromatographic resolution of the *S*-2-hydroxyacylglutathione products from α-oxo aldehyde, diamide derivatives, oxidised glutathione and phosphate salts (from the buffer). Diamide was initially added to crude samples of *S*-mandelylglutathione prior to chromatography but this compound was long retained on the column and was readily resolved from reduced (and oxidised) glutathione without further manipulation. The high retention volume of this compound may reflect hydrophobic interaction with the supporting matrix of the ion exchange resin.

Molecular Characteristics of S-2-*Hydroxyacylglutathiones*.—Characteristics of the ^{1}H and ^{13}C NMR spectra, FABS mass spectrometric analysis, specific optical rotations, optical purity, UV absorption spectra and TLC analysis of the *S*-2-hydroxyacylglutathione derivatives prepared are presented (Tables 1–6).

^{1}H and ^{13}C NMR spectra and FABS mass spectrometric analysis of the *S*-2-hydroxyacylglutathione derivatives prepared (Tables 1–3) were consistent with the assigned structures. Acylation of the glutathionyl thiol group gives rise to a marked magnetic inequivalence of the C-3 cysteinyl protons, indicating that there is lack of free rotation around the C(2)–C(3) and C(3)–S bonds and the methylene group is effectively locked in one conformation with respect to the rest of the molecule (*cf.* the ^{1}H NMR spectrum of oxidised glutathione[14]).

The stereospecificity of the glyoxalase I-catalysed formation of *S*-2-hydroxyacylglutathiones where there is a chiral centre at the 2-position in the acyl group (*S*-lactoyl-, *S*-glyceroyl- and *S*-mandelyl derivatives considered here) has been previously established only on the analytical scale and only for *S*-lactoylglutathione and *S*-mandelylglutathione. The product formed was exclusively *S*-D-lactoylglutathione [(*R*)-*S*-lactoylglutathione] and *S*-D-mandelylglutathione [(*R*)*S*-mandelylglutathione].[1,2,15] Moreover, the optical purity of *S*-2-hydroxyacylglutathione derivatives prepared on the preparative scale by the glyoxalase I-catalysed reaction is of concern since a previous report[16] where glyoxalase I and glyoxalase II were deployed in combination to prepare aldonic acids suggested that the stereospecificity of the reaction decreased with increasing scale of preparation. This may imply that the formation of *S*-lactoylglutathione, *S*-glyceroylglutathione and *S*-mandelylglutathione catalysed by glyoxalase I described herein produces a mixture of diastereoisomeric products through stereoinfidelity.

The configuration of the *S*-2-hydroxyacyl group in *S*-lactoylglutathione, *S*-glyceroylglutathione and *S*-mandelylglutathione was investigated by analysis of the corresponding aldonic acids produced by hydrolysis with glyoxalase II (chemical hydrolysis by hydroxide was not used to avoid hydroxide-catalysed racemisation; it is assumed that glyoxalase II does not alter the configuration of the 2-hydroxyacyl moiety, particularly in small scale analysis[1,2,14]). The configuration of lactic acid was investigated by enzymatic analysis with L-lactic dehydrogenase and D-lactic dehydrogenase.[17] The configurations of glyceric acid and mandelic acid were determined by optical rotation measurements of the purified aldonic acids with reference to authentic D- and L-isomers available commercially. Absolute configuration and optical purity data are presented in Table 4.

The glyoxalase I-catalysed reaction generally produced the (*R*)-*S*-2-hydroxyacylglutathione derivative: *S*-D-lactoylglutathione, *S*-L-glyceroylglutathione and *S*-D-mandelylglutathione. The optical purities of *S*-D-lactoylglutathione and *S*-D-mandelylglutathione were high > 97% without statistically significant optical impurity. However, there was significant optical impurity in *S*-L-glyceroylglutathione (optical purity 72 ± 10%). This may reflect a partial realignment of the substrate in the active site influenced by the proximity of the 3-hydroxymethyl group of the glyceroyl moiety to the hydrophobic region which accommodates the methyl and phenyl groups in the formation of *S*-D-lactoylglutathione and *S*-D-mandelylglutathione, respectively. This will be the subject of a further report to be published elsewhere.

The apparent poor stereospecificity of the glyoxalase enzymes in aldonic acid synthesis found by Whitesides and co-workers[16] may be due to racemisation of the product during sample treatment—boiling for an undisclosed time with barium carbonate, for example. However, it is conceivable that some (*S*)-*S*-hydroxyacylglutathione diastereoisomer may be formed by non-enzymatic reactions catalysed by phosphate dianion HPO_4^{2-} and other buffer ions.[18] The low concentrations of phosphate buffer and substrates. and the choice of pH has minimised the rate of the non-enzymatic reaction in our procedure.

Spectrophotometric data on the pure *S*-2-hydroxyacylglutathiones were also presented (Table 5). Particularly important were the extinction coefficients at 240 nm. The formation and hydrolysis of *S*-2-hydroxyacylglutathione is routinely followed by change in absorbance at this wavelength (by convention) to assay the activities of glyoxalase I and glyoxalase II respectively.[19–24] Several reports of these parameters have been quoted in the past where purity of the *S*-2-hydroxyacylglutathione was not well-defined.[1,2,21,25,26] Our re-evaluation provides standard coefficients in buffer systems routinely used in glyoxalase research: (a) 100 mmol dm^{-3} potassium phosphate buffer, pH 6.6 and (b) 50 mmol dm^{-3} Tris/HCl, pH 7.4.[19–26] Comparing these values with those previously reported, there is considerable agreement except for *S*-D-mandelylglutathione. The purity of the *S*-2-hydroxyacylglutathione was assessed (see Experimental section). All compounds prepared gave NMR spectra consistent with the absence of impurities, one spot by t.l.c. analysis (Table 6) and were unreactive towards DTNB. The yields for the preparations were: *S*-D-lactoylglutathione 91%, *S*-L-glyceroylglutathione 67%, *S*-D-mandelylglutathione 89% and *S*-glycolylglutathione 69%.

The recent demonstration of growth arrest and toxicity in human leukaemia HL60 cells *in vitro* induced by exogenous *S*-D-lactoylglutathione[11] has prompted an investigation of the structure–activity relationships between *S*-2-hydroxyacylglutathione derivative and possible anti-tumour activity. Such studies and allied research on the cellular regulation of the activity of glyoxalase II during cell proliferation and differentiation[19,20] require relatively large amounts of high purity *S*-2-hydroxyacylglutathiones. In this report, we have described simple, reliable and inexpensive procedures for the preparation of such compounds.

Table 4 Specific rotation, absolute configuration and optical purity of *S*-2-hydroxyacylglutathione derivatives

	Specific rotation, $[\alpha]_D^{14}$ [a]				Absolute configuration [b] of the acyl group	Optical purity [c] (%)
	$\bar{x}$	σ	r	n		
S-Glycolylglutathione	−22.73	0.17	0.9965	5	—	—
S-Lactoylglutathione	−7.50	0.08	0.9995	4	*R*-, D-	99 ± 3
S-Glyceroylglutathione	−7.42	0.18	0.9979	5	*R*-, L-	72 ± 10
S-Mandelylglutathione	−34.64	0.15	0.9985	4	*R*-, D-	97 ± 7

[a] Specific rotation $[\alpha]_D^{14}$ was calculated from $[\alpha]_D^{14} = 100 \cdot m/l$ where m is the x coefficient from linear regression of n optical activity measurements ( ) on c concentration of *S*-2-hydroxyacylglutathione derivative (g 100 ml^{-1}; range 0.40–0.66), where l is the optical pathlength (dm). Although units for $[\alpha]_D$ are recorded in ° for consistency with literature values, current IUPAC practice is to record the units as 10^{-1} deg cm^2 g^{-1}. Numerically the values are identical. [b] Absolute configuration was determined by enzymatic analysis (*S*-lactoylglutathione) or optical rotation of purified corresponding aldonic acids (*S*-glyceroyl- and *S*-mandelylglutathione). [c] Optical purity = %*R* − %*S*, calculated from enzymatic analysis (*S*-lactoylglutathione) or $[\alpha]_{obs}/[\alpha]_{max} \times 100$ (*S*-glyceroyl- and *S*-mandelyl glutathione).

Table 5 Spectrophotometric extinction coefficients of *S*-acylglutathione derivatives

	Potassium phosphate buffer, pH 6.6, 100 mmol dm^{-3}					Tris HCl buffer, pH 7.4, 50 mmol dm^{-3}				
	λ_{max}/nm	ε_{240} (mmol dm^{-3} cm^{-1})		ε_{254}		λ_{max}/nm	ε_{240} (mmol dm^{-3} cm^{-1})		ε_{254}	
		$\bar{x} \pm \sigma$	R^2	$\bar{x} \pm \sigma$	R^2		$\bar{x} \pm \sigma$	R^2	$\bar{x} \pm \sigma$	R^2
Reduced glutathione		0.100 ± 0.001	0.992	0.026 ± 0.001	0.918		0.137 ± 0.001	0.996	0.034 ± 0.001	0.976
S-Lactoyl-glutathione	233	3.38 ± 0.02	0.999	0.70 ± 0.01	0.998	233	3.36 ± 0.02	0.999	0.749 ± 0.002	0.999
S-Glycoly-gluthathione	231	3.21 ± 0.01	0.999	0.59 ± 0.01	0.998	231	3.28 ± 0.04	0.998	0.57 ± 0.01	0.997
S-Gluceroyl-glutathione	234	3.49 ± 0.03	0.999	0.82 ± 0.01	0.998	234	3.37 ± 0.02	0.999	0.74 ± 0.01	0.996
S-Mandelyl-glutathione	234	4.34 ± 0.05	0.997	1.59 ± 0.02	0.999	234	4.29 ± 0.05	0.087	1.46 ± 0.01	0.999

Extinction coefficients were calculated by linear regression of 24 absorbance-concentration data pairs.

Table 6 Chromatographic R_f values of *S*-2-hydroxyacylglutathiones*

Compound	Solvent 1 R_f ± SEM	Solvent 2 R_f ± SEM	Solvent 3 R_f ± SEM
Reduced glutathione	0.57 ± 0.05	0.19 ± 0.01	0.09 ± 0.02
Oxidised glutathione	0.33 ± 0.03	0.15 ± 0.01	0.04 ± 0.01
S-Glycolylglutathione	0.59 ± 0.05	0.34 ± 0.01	0.17 ± 0.01
S-Lactoylglutathione	0.61 ± 0.04	0.39 ± 0.01	0.20 ± 0.01
S-Glyceroylglutathione	0.52 ± 0.03	0.30 ± 0.01	0.32 ± 0.02
S-Mandelylglutathione	0.70 ± 0.03	0.53 ± 0.01	0.32 ± 0.02

* Chromatography was performed on silica gel 60 plates in triplicate with developing solvents: 1, Propanol–acetic acid–water, 12:10:5; 2, Butanol acetic acid water, 10:5:5; 3, Pentanol–acetic acid–water, 10:5:5. Detection by ninhydrin in ethanol (0.2%).

Previous reports on the preparation of *S*-2-hydroxyacylglutathione derivatives for biological studies have employed glyoxalase I-catalysed reactions but have described the preparation of only unpurified products,[21] purification procedures with poor resolution[22] or have developed a procedure for *S*-lactoylglutathione only using unbuffered reaction mixtures (*S*-lactoylglutathione hydrolyses spontaneously above pH 7.4).[23] Glyoxalase I has also been bound to Sepharose 4B for ease of separation from the product but this decreases the effective activity of the enzyme.[24] Here, we have demonstrated that the enzymatic formation of *S*-2-hydroxyacylglutathiones by glyoxalase I can be successfully and reliably deployed on the gram-scale to prepare *S*-D-lactoylglutathione and analogues of high optical purity from methylglyoxal and other α-oxo aldehyde substrates in a phosphate-buffered reaction mixture.

The procedures described here for the preparation of *S*-2-hydroxyacylglutathiones and re-evaluated spectrophotometric data should assist future developments in the increasingly important and expanding pharmacology of *S*-D-lactoylglutathione and analogous compounds.

Experimental

Materials.—Methylglyoxal (MeCOCHO), dihydroxyacetone, diamide [1,1′-azo(*N*,*N*-dimethylformamide)], 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glyoxalase I (grade X, lyophilised powder from yeast), glyoxalase II (lyophilised powder from beef liver) and reduced glutathione were purchased from Sigma Chem. Co. Ltd. (Poole, Dorset, UK). Glyoxal $(CHO)_2$ (40% aqueous solution) was obtained from Fluka (Glossop, Derbyshire, UK), and phenylglyoxal (PhCOCHO) was purchased from Aldrich Chem. Co. Ltd. (Poole, Dorset, UK). Hydroxypyruvaldehyde ($HOCH_2COCHO$) was synthesised from dihydroxyacetone by the method of Reeves and Ajl.[27] Diaflo PM10 ultrafiltration membranes were purchased from Amicon (Stonehouse, Gloucester, UK). L-Lactic dehydrogenase, D-lactic dehydrogenase, D-glyceric acid, L-glyceric acid, D-mandelic acid and L-mandelic acid were purchased from Sigma.

Synthesis of S-2-*Hydroxyacylglutathione Derivatives.*—Reduced glutathione (922 mg, 3 mmol), α-oxo aldehyde (3 mmol) and glyoxalase I (90 units) in sodium phosphate buffer (5 mmol dm^{-3}; 300 cm^3), pH 6.6, were incubated in a shaking water-bath at 37 °C. Samples (100 mm^3) were taken periodically, diluted to 1 cm^3, and assayed for *S*-2-hydroxyacylglutathione content by monitoring the decrease of absorbance at 240 nm upon addition of glyoxalase II (1 unit).

$$RCH(OH)CO\text{-}SG + H_2O \xrightarrow{\text{Glyoxalase II}} RCH(OH)CO_2H + GSH$$

The concentration of *S*-2-hydroxyacylglutathione was deduced using the appropriate change in extinction coefficient for the above reaction. The incubations were continued until the formation of *S*-2-hydroxyacylglutathione reached a maximum. The reaction mixture was then filtered through a PM10 membrane and the glyoxalase I collected was washed with buffer and reclaimed for further incubations. The filtrate containing the product was lyophilised to dryness.

Purification of S-2-*Hydroxyacylglutathione Derivatives by Ion Exchange Chromatography.*—*S*-2-Hydroxyacylglutathiones were purified by anion exchange chromatography on Dowex 1 in the formate form. The column (5 × 30 cm) was initially equilibrated with 122 mmol dm^{-3} formic acid (*S*-lactoylglutathione and *S*-mandelylglutathione) or 22 mmol dm^{-3} formic acid (*S*-glycolylglutathione and *S*-glyceroylglutathione). Lyophilised preparations of *S*-2-hydroxyacylglutathione were dissolved in formic acid (122 mmol dm^{-3}; 9 cm^3). Diamide in methanol (2 mol dm^{-3}) was added dropwise to oxidise any contaminating reduced glutathione remaining from incomplete conversion into the thioester product (*S*-2-hydroxyacylglutathiones are often better resolved from oxidised rather than reduced glutathione on Dowex 1[23,25]). The pH of the sample was decreased to that of the equilibrated column (with 90% formic acid) and was loaded on to the column at 150 cm^3 h^{-1}. The column was eluted with linear gradients of formic acid at a flow rate of 300 cm^3 h^{-1}. The eluate was monitored at 254 nm and the peaks of absorbance collected by a FRAC 200 fraction collector (Pharmacia). Fractions for a peak of absorbance were pooled and lyophilised to dryness.

Characterisation of S-2-*Hydroxyacylglutathiones and Criteria of Purity.*—*S*-2-Hydroxyacylglutathiones were characterised by ^1H and ^{13}C NMR spectroscopy, FABS mass spectrometric analysis and UV absorption spectroscopy. The purity of these compounds was analysed: (i) by TLC on silica gel in several developing solvent systems with detection by ninhydrin, (ii) by lack of reactivity with DTNB in 100 mmol dm^{-3} sodium phosphate buffer, pH 7.4, monitored by increase in absorbance at 412 nm; $\Delta\varepsilon$ = 13.6 mmol dm^{-3} cm^{-1} for thiol groups,[28] and (iii) from the ^1H and ^{13}C NMR spectra.

The extinction coefficients for *S*-acylglutathiones are often deployed and quoted in relation to the assay of activities of glyoxalase enzymes. Since there is some variation in the reported values for preparations of *S*-2-hydroxyacylglutathione where the purity was not defined, these coefficients have been re-evaluated. They were determined at 240 nm by measuring the absorbance of a minimum of 6 concentrations in quadruplicate such that the absorbance values were in the range 0–1.0 A.U. Linear regression was performed on plots of absorbance *versus* concentration. Extinction coefficients (slopes) with standard deviation are given for determinations in 100 mmol dm^{-3} potassium phosphate buffer, pH 6.6, and 50 mmol dm^{-3} Tris/HCl, pH 7.4, the standard buffer systems used in assay of glyoxalase I and glyoxalase II activity respectively.

Optical Purity and Specific Rotation.—Specific rotation values, $[\alpha]_D$, for *S*-2-hydroxyacylglutathione derivatives were determined in aqueous solution over the concentration range 0.40–0.67 g 100 cm^{-3} in water. Values for $[\alpha]_D$ were calculated from least squares linear regression of the observed optical rotation α (°) on the concentration of *S*-2-hydroxyacylglutathione derivative (g 100 cm^{-3}); $[\alpha]_D$ 100 m/l where m is the x coefficient from linear regression and l is the optical pathlength (dm).

The optical purity and absolute configuration of acyl groups in *S*-lactoylglutathione, *S*-glyceroylglutathione and *S*-mandelylglutathione were determined by analysis of the configuration of the corresponding aldonic acid after hydrolysis with glyoxalase II in 50 mmol dm^{-3} Tris-HCl, pH 7.4. Glyoxalase II was then removed by ultrafiltration.

The configuration of lactic acid produced from *S*-lactoylglutathione was determined by enzymatic assay with D-lactic dehydrogenase, and L-lactic dehydrogenase, in the presence of hydrazine by methods similar to those previously described by Beutler.[29] Assay solutions contained 742 mmol dm^{-3} glycine, 285 mmol dm^{-3} hydrazine sulphate, 1.5 mmol dm^{-3} DETAPAC, 1 mmol dm^{-3} NAD^+, 25 units cm^{-3} lactic dehydrogenase, pH 9.2, and *ca.* 0.085 mmol dm^{-3} lactic acid equivalent. The formation of NADH to endpoint was followed by spectrophotometric measurement at 340 nm. The concentration of D-lactic acid and L-lactic acid present was deduced by reference of the endpoint absorbance of the test solution to those of standard D-lactic acid and L-lactic acid solutions. D-Lactic dehydrogenase and L-lactic dehydrogenase were completely stereospecific in their reactivity with D- and L-lactic acid standards.

The configuration of glyceric acid and mandelic acid were determined by optical rotation measurements of the purified aldonic acids produced from hydrolysis of *S*-glyceroylglutathione (290 mg) and *S*-mandelylglutathione (60 mg). Glyceric acid was purified by anion exchange chromatography on Dowex 1, formate form eluting with 30 mmol dm^{-3} formic acid. Glyceric acid was detected by flow spectrophotometry at 254 nm (weak absorbance). Mandelic acid was purified by preparative reverse phase HPLC using a radial compression cartridge (10 × 2.5 cm) of octadecylsilica (ODS) with a mobile phase of 10 mmol dm^{-3} formic acid. The flow rate was 10 cm^3 min^{-1}. Mandelic acid was detected by flow spectrophotometry at 220 nm; the retention time of mandelic acid was 7.2 min. Optical rotation measurements of purified glyceric acid (*c* 0.61 g 100 cm^{-3}) and mandelic acid (*c* 0.047 g 100 cm^{-3}) and authentic L-glyceric acid and D-mandelic acid were recorded. The specific rotation $[\alpha]_D^{14}$ for authentic L-glyceric acid and D-mandelic acid were −17.2 and −154, respectively.

Acknowledgements

J. D. C. and P. J. T. thank the Cancer Research Campaign for research funding. This is a contribution from the University of Essex, Glyoxalase Research Group.

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Paper 1/02845F
Received 13*th June* 1991
Accepted 31*st July* 1991