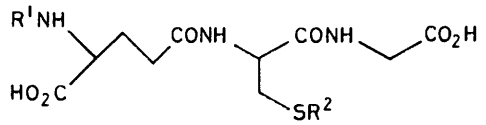
Photoaffinity Labels for Enzymes Capable of Binding Glutathione or its Disulphide

By **Claudius D'Silva, Andrew P. Seddon,** and **Kenneth T. Douglas,*** Department of Chemistry, University of Essex, Colchester CO4 3SQ

The syntheses and photolyses of several glutathione analogues incorporating aryl azide moieties on the N-terminus or SH sites are described. Such labels include S-blocked glutathiones, N,S-blocked glutathiones and an N,N'-blocked glutathione disulphide. *S*-(4-Azidophenacyl)glutathione has been used to label covalently yeast glyoxalase I, beef liver glyoxalase II, and glutathione-*S*-transferase; *N,S*-bis(4-azidobenzoyl)glutathione has been used to label covalently formaldehyde dehydrogenases from *Pseudomonas* species and beef liver; *N,N'*-bis-(4-azidobenzoyl)glutathione disulphide has been successfully used to label covalently yeast glutathione reductase.

GLUTATHIONE (1; GSH) plays a myriad of roles in biochemistry, some well-understood and others undoubtedly undescribed, as yet. Thus, for example, it functions in several types of enzyme-catalysed reactions as coenzyme (*e.g.* formaldehyde dehydrogenase), cosubstrate (*e.g.* glyoxalase I, glutathione-*S*-transferase), or as a part of the substrate architecture (glyoxalase II). It is also vital to a variety of life processes including the detoxification of xenobiotics, maintenance of SH levels of proteins, disulphide-exchange processes, removal of hydrogen peroxide, organic peroxides, and free radicals and possibly in amino-acid transport across membranes, as well as other phenomena.[1-3] Glutathione disulphide, the oxidised form of GSH, is the substrate for glutathione reductase and may function, *inter alia*, in the regulation of the hexose monophosphate pathway in the erythrocyte and in oxidative phosphorylation.[4]



(1) $R^1 = R^2 = H$
(2) $R^1 = H$, $R^2 = CH_2COC_6H_4N_3$-*p*
(3) $R^1 = H$, $R^2 = C_6H_3(2\text{-}NO_2,4\text{-}N_3)$
(4) $R^1 = R^2 = COC_6H_4N_3$-*p*
(5) $R^1 = COC_6H_4N_3$-*p*, $R^2 = CH_2C_6H_4Br$-*p*
(6) $R^1 = COC_6H_4N_3$-*p*, R^2 = S-glutathione, *N*-$COC_6H_4N_3$-*p*

The technique of photoaffinity labelling offers a tool of powerful versatility in modern biochemistry. Already applications for this approach have flourished in studies of enzyme active-sites, protein binding-sites, receptor sites, transport systems, cell organelles and assemblies.[5,6] This, coupled with the importance of glutathione in one of its forms (free GSH, S-blocked glutathione, or as the oxidised form, glutathione disulphide) have led us to synthesise and characterise a battery of photolabels based on the glutathione structure. Applications of one of these to a variety of enzyme systems have been described recently.[7,8] However, in view of the potential usefulness of such compounds to a wide variety of problems we felt it timely to describe the details of the synthesis, purification, and photolytic properties of a wide structural range of such derivatives.

EXPERIMENTAL

Photoirradiation of solutions was effected by means of a Quantum Yield Photoreactor (Model 2001, Applied Photophysics) with a 250-W medium-pressure mercury lamp. Photolyses were performed in quartz cuvettes (1-cm path-length), thermostatted at an appropriate temperature (usually 20—25 °C). Photolysis apparatus components were mounted on an optical rail allowing photolyses to be carried out at various source-sample distances, commonly 15—25 cm. A *ca.* 300—350 nm 'wavelength window' of the emitted radiation was selected by means of a 3-compartment, quartz chemical filter cell (3 × 2-cm path-length) containing appropriate solutions (*viz* 1.71M-$NiSO_4$, 1.0M-$CoSO_4$, and 0.0133M-$SnCl_2$).[9] All filter solutions were pre-irradiated (1 h) and transmission values determined as described.[9]

Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of Manchester. Precursors and solvents were purchased commercially and purified appropriately.

U.v. and visible spectra were recorded by means of a Pye-Unicam SP8-100 or Carlo Erba Spectracomp 601 spectrophotometer at 25 °C. I.r. spectra were obtained using a Pye-Unicam SP-200 spectrophotometer and ^1H n.m.r. spectra using a Varian EM 360 instrument. Chemical shifts (δ) are in p.p.m. from $SiMe_4$; resonances marked with an asterisk disappeared on addition of D_2O; spectra were run in [$^2H_6$]dimethyl sulphoxide. Thin layer chromatography (t.l.c.) was performed using 0.2 mm precoated plates (Merck DC-Plastikfolien Kieselgel 60 F_{254}). Solvents used were: A, n-butanol–acetic acid–water (7 : 1 : 2); B, phenol–water (4 : 1); C, water–n-butanol–ethanol (5 : 4 : 1). Spots were visualized by u.v. illumination and ninhydrin–acetone spray. Tests for SH and NH_2 blocking were carried out by means of Ellman's [10] and ninhydrin reagents, respectively. Melting points (uncorrected) were determined with a m.p. microscope (Reichart).

4-Azidoacetophenone [11a] and 4-azidophenacyl bromide [11b] were prepared by literature methods. During all syntheses involving derivatives with azido-groups the systems were protected from light, usually by metal foil.

S-(4-*Azidophenacyl*)*glutathione* (2).—This compound was prepared by a modification of a literature procedure,[12] described in detail below. To a stirred solution of reduced glutathione (0.614 g, 0.2 mmol) in sodium hydroxide (4 cm³, 1M-NaOH), ethanol (12—15 cm³) was added to the cloud point. 4-Azidophenacyl bromide (0.480 g, 0.002 mol), dissolved in the minimum volume of ethanol was

added dropwise to the stirred reaction mixture at ambient temperature. Additional ethanol and/or sodium hydroxide solution were added if precipitation occurred [addition of an *excess* of sodium hydroxide solution at this stage caused the solution to change in colour from pale yellow to orange-amber yielding an as yet unidentified product and very poor yield of (2)]. The reaction mixture was stirred (4 h) under nitrogen at ambient temperature, acidified (pH 3.5), chilled, and the precipitated product collected by vacuum filtration. (The chilling time required for product-precipitation was usually between 3 and 24 h). The product was washed (H_2O), air-dried, and recrystallized ($\times 2$) from water–ethanol, the temperature being kept at or below 60 °C, to give a pale-yellow solid (0.31 g, 65%), m.p. 186 °C with decomposition (Found: C, 46.8; H, 5.1; N, 17.9. $C_{18}H_{22}N_6O_7S$ requires C, 46.4; H, 4.8; N, 18.0%): t.l.c. R_F (solvent A) 0.27; R_F (solvent B) 0.24: $\lambda_{max.}$ (1.6% v/v DMSO, pH 6.60, 0.1M-KH_2PO_4 buffer) 297 nm (log ε 4.29): ν(N_3) (NaCl) 2 090 cm^{-1}: δ [$(CD_3)_2SO$–D_2O] 8.0 (2 H, d, ArH), 7.2 (2 H, d, ArH), 4.4 (1 H, m, CH), 3.7 (2 H, s, CH_2COAr), 3.4 (1 H, m, CH), 2.8 (2 H, s, CH_2), and 2.5 and 1.9 (6 H, m, $3 \times CH_2$).

S-(4-*Azido*-2-*nitrophenyl*)*glutathione* (3).—4-Fluoro-2-nitrophenyl azide (0.314 g, 1.7 mmol) and reduced glutathione (0.530 g, 1.7 mmol) were coupled as above. The product was similarly recrystallized from water–ethanol to give a bright yellow solid (0.33 g, 40%), m.p. 198—200 °C with decomposition (Found: C, 40.8; H, 4.0; N, 20.4. $C_{16}H_{19}N_7O_8S$ requires C, 40.9; H, 4.1; N, 20.9%), $\lambda_{max.}$ (1.6% Me_2SO, pH 6.60, 0.1M-KH_2PO_4) 266, 282sh, and 402 nm (log ε 4.24, 4.15 and 3.29); ν(N_3) (NaCl) 2 090 cm^{-1}; δ [$(CD_3)_2SO$–D_2O] 7.6 (3 H, m, ArH), 4.6 (1 H, m, CH), 3.5 (3 H, m, CH_2 and CH), 2.6 (2 H, s, CH_2), and 2.4 and 2.1 (4 H, 2 m, Glu-CH_2CH_2); t.l.c. R_F (solvent A) 0.27; R_F (solvent B) 0.24.

N,S-Bis-(4-*azidobenzoyl*)*glutathione* (4).—To an ice-cooled, stirred solution of reduced glutathione (5 g, 0.0163 mol) in sodium hydroxide solution (16.2 cm^3; 2M) was added, during 30 min, 4-azidobenzoyl chloride [prepared from 4-azidobenzoic acid (5.84 g, 0.034 mol)] in benzene (20 cm^3) whilst the pH was simultaneously maintained at 9.5 by dropwise addition of sodium hydroxide (2M). After 4 h at 0 °C and 12 h at ambient temperature the solution was acidified (pH 4.5), extracted with ether (3×100 cm^3), acidified to pH 2, and the product collected by filtration. The aqueous layer was then extracted with ethyl acetate, washed with water, and dried ($MgSO_4$). Solvent was evaporated off and the residue combined with the earlier-isolated product, dried, and recrystallised from ethanol to give a pale yellow solid (3.26 g, 33.8%), m.p. 159—161 °C with decomposition. The compound was further recrystallised from methyl cyanide for elemental analysis (Found: C, 48.0; H, 4.0; N, 20.6; S, 5.5. $C_{24}H_{23}N_9O_8S$ requires C, 48.2; H, 3.9; N, 21.1; S, 5.4%), $\lambda_{max.}$ (0.8% DMSO, pH 6.60, 0.1M-KH_2PO_4 buffer) 276 nm (log ε 4.46); ν(N_3) (NaCl) 2 110 cm^{-1}; δ [$(CD_3)_2SO$] 8.7* (1 H, d, NH), 8.2* (2 H, d, 2 × NH), 7.9 (4 H, d, ArH), 7.1 (4 H, d, ArH), 4.4 (1 H, m, CH), 3.7 (2 H, d, CH_2-S), 3.2 (1 H, m, CH), 2.4 (2 H, s, Gly CH_2), and 2.2 (4 H, m, Glu-CH_2-CH_2); t.l.c., R_F (solvent A) 0.34; R_F (solvent C) 0.29, [GSH R_F (solvent A) 0.05, R_F (solvent B) 0.18].

N-(4-*Azidobenzoyl*)-S (4-*bromobenzyl*)*glutathione* (5).—To a stirred solution of *S*-(4-bromobenzyl)glutathione [12] (2.5 g, 5.25 mmol) in sodium hydroxide (1M) and an equal volume of dioxan (peroxide free), was added a slurry containing 4-azidobenzoyl chloride (1.05 g, 5.8 mmol) and 4-dimethylaminopyridine (0.705 g, 5.8 mmol) in dioxan–ether, pH_{app} adjusted to 9.5 and the mixture stirred overnight. A further equivalent of 4-azidobenzoyl chloride and 4-dimethylaminopyridine was added to maintain a solution pH_{app} of 9.5. After being stirred for 16 h, the solution was acidified (pH 7.0), extracted once with ether, and then with ethyl acetate (the pH being simultaneously adjusted to 2); the extracts were washed with water, dried (Na_2SO_4), evaporated, and the residue recrystallised ($\times 2$) from ethyl acetate–methanol to give a colourless solid (1.7 g, 55%), m.p. 143—145 °C. The compound was further purified by LH-20 Sephadex (1.1 × 42 cm) chromatography with MeOH as eluant, to yield material of m.p. 138—140 °C (Found: C, 46.9; H, 4.2; N, 13.2. $C_{24}H_{25}BrN_6O_7S$ requires C, 46.4; H, 4.1; N, 13.5%), ν(N_3) (NaCl) 2 190 cm^{-1}; δ [$(CD_3)_2SO$] 8.6* (1 H, d, NH), 8.2* (2 H, d, NH), 7.9 (2 H, d, ArH), 7.2 (6 H, m, ArH), 4.3 (1 H, m, CH), 3.6 (4 H, s, $2 \times CH_2$), 3.3 (1 H, m, CH), and 2.4 and 2.1 (6 H, m, $3 \times CH_2$). T.l.c. R_F (solvent A) 0.6; R_F (solvent C) 0.18; [*S*-(4-bromobenzyl)glutathione; R_F (solvent A) 0.31, R_F (solvent B) 0.27, R_F (solvent C) 0.23)]: $\lambda_{max.}$ (0.8% Me_2SO, pH 6.6, 0.05m-KH_2PO_4 buffer) 271 nm (log ε 4.32).

N,N-*Bis*-(4-*azidobenzoyl*)*glutathione Disulphide* (6).—To a stirred solution of glutathione disulphide (3 g, 4.9 mmol) in sodium hydroxide (2M) and an equal volume of dioxan (peroxide-free) was added a slurry containing 4-azidobenzoyl chloride (0.98 g, 5.4 mmol) and 4-dimethylaminopyridine (0.66 g, 5.4 mmol) in dioxan–ether; the pH_{app} was adjusted to *ca.* 10 and the mixture stirred overnight. A further equivalent of 4-azidobenzoyl chloride and dimethylaminopyridine was added to maintain the pH_{app} *ca.* 10. After being stirred for 24 h the solution was acidified (pH 4.5), extracted once with ether, and finally with ethyl acetate (with propan-1-ol as necessary) the pH being simultaneously adjusted to 2; the extracts were washed with water, dried (Na_2SO_4), and evaporated, and the residue recrystallised from methanol–ether to give an off-white solid (1.97 g, 44.4%), m.p. 127—129 °C. Colourless crystals were obtained by chromatography (1 g) on Sephadex LH-20 (1.1 × 50 cm) (methanol eluant). Fractions containing R_F(A) 0.3 were pooled and evaporated to give an oil which was dissolved in the minimum quantity of hot methanol–ethyl acetate and refrigerated to give a colourless solid (0.77 g, 77%), m.p. 130—132 °C (Found: C, 44.0; H, 4.3; N, 17.9; S, 7.3. $C_{34}H_{38}N_{12}O_{14}S_2H_2O$ requires C, 44.35; H, 4.4; N, 18.25; S, 7.0%); $\lambda_{max.}$ (0.8% Me_2SO, pH 7.6, 0.06M-NaH_2PO_4), 272 nm (log ε 4.55); ν(N_3) (NaCl) 2 100 cm^{-1}; δ [$(CD_3)_2SO$] 8.6* (2 H, d, NH), 8.1* (4 H, d, 4 × NH), 7.8 (4 H, d, ArH), 7.1 (4 H, d, ArH), 4.3 (2 H, m, 2 × CH), 3.6 (4 H, d, 2 × CH_2S), 2.9 (2 H, m, 2 × CH), 2.4 and 2.0 (12 H, m, 2 × Gly-CH_2, 2 × Glu CH_2-CH_2). T.l.c. R_F (solvent A) 0.3, R_F (solvent C) 0.20.

RESULTS

The photolyses of compounds (2)—(6) were studied as described under various conditions. The spectral changes resulting from the photolysis of compound (2) (2.16×10^{-3}M), when irradiated 15 cm from a source of incident light of wavelength range *ca.* 300—*ca.* 350 nm, are shown in Figure 1. Over the first 50—60 min the spectrum changes smoothly, the peak at 296 nm disappearing, with reasonably tight isosbestic points at *ca.* 260 and *ca.* 325 nm. After 60 min, a slower process is evident with consequent shifts in the isosbestic wavelengths. For the faster process good

first*-order kinetics are obeyed as can be seen from the rate plot overlaid on the same axes: the half-life under these conditions was 12.8 min ($k_1 = 0.054$ min^{-1}). When the concentration of compound (2) was decreased to 7.40×10^{-5}M and the photolysis repeated at pH 7.0 (phosphate buffer) at 20 °C, the value of k_1 was found to be 5.15 min^{-1} (corresponding to $t_{\frac{1}{2}} = 8$ s). Thus, decreasing the concentration of compound (2) by some 300-fold, increases

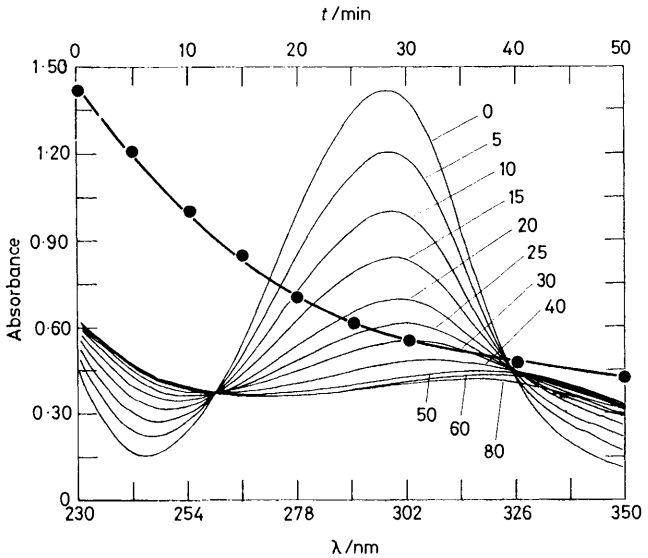


FIGURE 1 Spectral changes of *S*-(4-azidophenacyl)glutathione (2.16×10^{-3}M) 15 cm from light source at 20.0 °C (pH 7.0) as described in the text: spectra were determined on aliquots (100 μl) transferred from the photolysis cuvette at times (in min) indicated to pH 7.50 phosphate buffer. The overlaid line shows the corresponding time course of the reaction following absorbance changes at 300 nm as a function of photolysis time: points are experimental, from the main figure; the solid line was obtained by non-linear regression analysis of the data using the exponential form of the first-order rate equation with $k = 0.054$ min^{-1}, and initial and final absorbances of 1.426 and 0.341, respectively.

the apparent rate of photolysis by *ca.* 100-fold. More efficient photolysis of compound (2) is therefore achieved by using as low a concentration of (2) as possible, within the limits set by the binding constant for the biological glutathione-binding site concerned and its inherent photostability. In general, avoiding oversaturation of target sites by photoaffinity labels is to be recommended to minimize non-specific labelling problems. Apparent rates of photolysis of (2) (7.57×10^{-5} M) at various pH values were (pH, k_1): 7.84, 1.06×10^{-2} s^{-1}; 7.06, 1.21×10^{-2} s^{-1}; 5.98, 1.04×10^{-2} s^{-1}; 5.06, 1.06×10^{-2} s^{-1}. Thus, within the usual physiologically interesting pH range, the rate of photolysis of compound (2) is effectively independent of pH.

Derivative (3), the nitroaryl azide, was also photolysed smoothly on irradiation with incident light of wavelength *ca.* 300—*ca.* 350 nm, at pH 6.60. A reasonable fit to the first-order rate equation was obtained, for a half-life of *ca.* 2 min, under the conditions used. During photolysis the absorbance at 263 nm (and shoulder at *ca.* 280 nm) decreased and a new weak band appeared at *ca.* 365 nm.

Derivative (4) was photolysed rapidly when irradiated at 25 cm from the light source with incident radiation in the wavelength region *ca.* 300—350 nm. From Figure 2 (a) it is

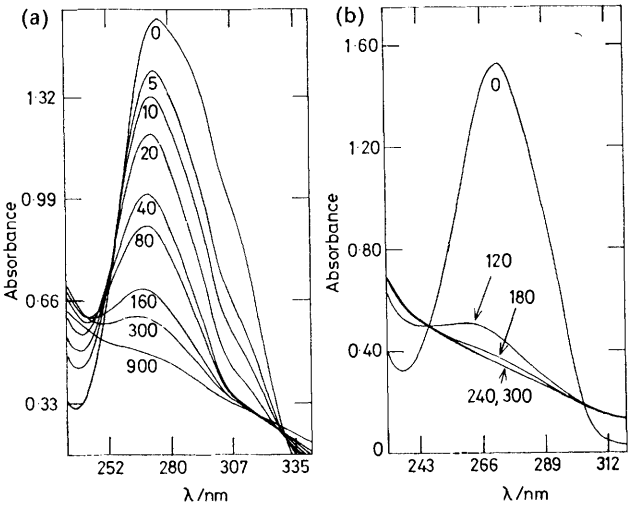


FIGURE 2 (a) Repetitive spectral scans of *N*,*S*-bis-(4-azidobenzoyl)glutathione (4) photolysed 25 cm from the light source at 22 °C with incident radiation of *ca.* 300—350 nm. The spectra are annotated with the photolysis times in seconds. The photolysis medium was 0.1M-potassium phosphate buffer, pH 6.60. (b) Repetitive spectral scans of *N*,*S*-bis-(4-azidobenzoyl)glutathione (4) photolysed 15 cm from the light source at 22 °C with incident radiation of *ca.* 300—350 nm. The spectra are annotated with the photolysis times in seconds. Photolyses were effected in 0.1M-sodium pyrophosphate buffer, pH 8.0.

apparent that the time-course of the photolysis is complex, no sharp isosbestic points being obtained on repetitive spectral scanning of the reaction mixture of compound (3) photolysed under the above conditions at pH 6.60 (0.1M potassium phosphate buffer). In addition to the shift of the peak at *ca.* 276 nm to lower wavelengths during the course of the photolysis, the shoulder at *ca.* 310 nm disappears. By plotting the A_{280} *versus* time for this reaction, it was found that there is an initial rapid phase and a slower phase. Whilst it is difficult to quantitate the rate of photolysis it is clear that a high percentage of compound (4) is photolytically destroyed in the first few minutes under these irradiation conditions, making it a very labile reagent. When photolysed under similar conditions to the above, but closer to the light source (*i.e.* at 15 cm) clean isosbestic points were apparently obtained [see Figure 2(b)] and over 90% photolysis occurred in 2 min, the reaction being effectively complete in 4 min.

N-(4-Azidobenzoyl)-*S*-(4-bromobenzyl)glutathione (5) was photolysed readily at pH 6.60 with incident light of wavelengths *ca.* 300—*ca.* 350 nm but the time course was not smooth, although it was close to first-order behaviour. Photolytic changes in the spectrum were complete in *ca.* 10 min at 25 cm from the light source but the poor isosbestic points indicated a complex process.

The photoaffinity-analogue of oxidised glutathione [(6) *N*,*N*′-bis-(4-azidobenzoyl)glutathione disulphide] was smoothly photolysed, with good isosbestic points (*ca.* 248 and *ca.* 302 nm) on irradiation using the chemical combination filter as used above to transmit a ' window ' of light of

* Although the time course of the spectral changes obeys the first-order rate equation ($y = ce^{-kt}$) no kinetic inferences are intended at this stage. However, the fit to the first-order equation allows prediction of the percentage spectral change which will have occurred in any given time (relative to a known half-life), obviously useful in the design of photoaffinity-labelling experiments.

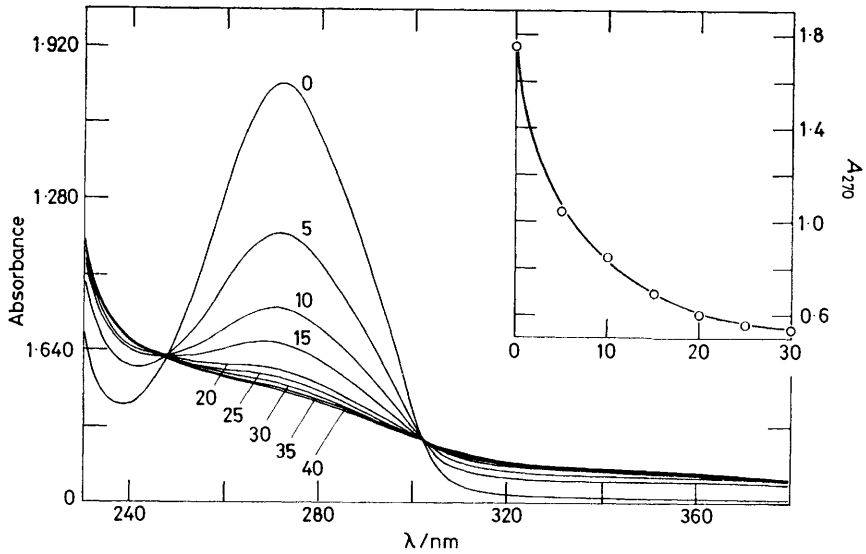


FIGURE 3 Spectral changes in pH 7.6 (0.06M) phosphate buffer for (6), *N,N'*-bis-(4-azidobenzoyl)glutathione disulphide on photolysis at 22 °C, 25 cm from a medium-pressure mercury lamp with incident light of wavelengths between *ca.* 300 and *ca.* 350 nm, photolysis times (minutes) being indicated on the spectra. The inset shows the time-course of the photolysis by recording the absorbance at 270 nm as a function of photolysis time. The points are experimental, from the main figure, the line is theoretical for a first-order process of rate constant 0.132 min^{-1} and initial and final absorbances of 1.746 and 0.510, respectively and was obtained by non-linear regression analysis (least-squares) using the first-order exponential equation.

wavelengths between *ca* 300 and *ca.* 350 nm (see Figure 3). Photolysis of compound (6) followed good first-order kinetics (see inset to Figure 3) with half-lives of 3.2 and 5.2 min when irradiated in pH 7.60 phosphate buffer (0.06M) 15 and 25 cm from the light source, respectively. Consequently, for photoaffinity-labelling experiments using compound (6), irradiation at 15 cm would yield *ca.* 90% photolysis after 10 min.

DISCUSSION

The GSH molecule (1) although polyfunctional is most amenable to synthetic manipulations *via* the NH_2 and SH sites. By modification of one or both of these, a number of photolabile aryl azide derivatives is readily available. Similarly, the *N,N'*-bis(4-azidobenzoyl) derivative of GSSG is now available. These reagents should be of wide usefulness in view of the enormous range of biochemical systems in which GSH and/or GSSG is functional, *e.g.* coenzymic functions, transport phenomena.

Derivatives (2) and (3) are S-blocked glutathiones and of this pair we have found (2) to be a successful photoaffinity label for a number of glutathione-dependent systems. Compound (3) suffers, relative to (2), from solubility limitations. *S*-(4-Azidophenacyl)glutathione (2) has been found to be a competitive inhibitor of yeast glyoxalase I ($K_i = 1.05 \times 10^{-4}$M) and when irradiated with this enzyme at a concentration of $30K_i$, led to 64% covalent inhibition.* Essentially, quantitative protection against this photoinactivation was afforded by the competitive inhibitor of glyoxalase I, *S*-(4-bromobenzyl)gluthathione.[7] With glyoxalase II, the K_i (competitive) for (2) is 7.96×10^{-4}M, and covalent inhibition to a level of $42 \pm 5\%$ can be achieved by photoactivation at a level of $4K_i$; the covalent inhibition is blocked by the GSH-methylglyoxal hemimercaptal adduct, a competitive inhibitor of this enzyme.[8] In addition, compound (2) at 1.67 mM on photoactivation leads to $86 \pm 5\%$ covalent inhibition of crude beef liver glutathione-*S*-transferase (the K_i for the photostable analogue *S*-(4-bromobenzyl)glutathione was found to be 1.1×10^{-5}M).[8] *S*-(4-Azidophenacyl)glutathione, (2), also inhibits beef liver formaldehyde dehydrogenase (20% inhibition at 0.8 mM) but the inhibition is paraboidal; a similar weak, paraboidal inhibition of this enzyme occurs for compound (3), 10% inhibition being attained at 0.93 mM.[13] There is essentially no inhibition of yeast glutathione reductase by compounds (2) or (3).[14] Both compounds (2) and (3) compete with γ-glutamyl 4-nitroanilide–glycylglycine in the assay for porcine kidney γ-glutamyl transpeptidase. However, photoinactivation was irreproducible, probably because compounds (2) and (3) behaved as competitive substrates.[15]

N,S-Bis-(4-azidobenzoyl)glutathione, (4), is an extremely photosensitive *bifunctional* photoaffinity label, photolysis being essentially complete in 4 min, under appropriate conditions. The high photosensitivity is very useful in view of the (unpredictable) photosensitivity of native proteins as it allows a short exposure time to the incident light. In addition the bifunctionality

* Covalent inhibition was generally ascertained by photolysis of the enzyme in the presence (experimental) and absence (control) of the appropriate azide followed by passage over G-25 or G-50 to separate protein from reversibly bound inhibitor.[7,8] Protection experiments were effected similarly but with a powerful competitive inhibitor present at high levels in the initial incubation mixtures of both control and experimental runs.[7,8] Protection against azide-originated photoinactivation by a competitive inhibitor is often taken as evidence that the labelling has occurred at the active site although strictly this is not necessarily so, *e.g.* rapidly equilibrating conformationally distinct enzyme forms could exist.

offers a series of major advantages over a monofunctional analogue. The most obvious is its cross-linking potential: cross-linking could occur between adjacent protein molecules in an oligomer, from a protein to its immediate biological surrounding (membranes, other proteins *etc.*) or within a single protomer (in this case linking parts of the same of distinct polypeptide chains). We have found that compound (4) is an effective competitive inhibitor of yeast glyoxalase I with $K_i = 6.7 \times 10^{-5}$M, although to date no covalent incorporation experiments have been attempted.[16] It also competitively inhibits formaldehyde dehydrogenase from *Pseudomonas* species ($K_i = 1.19$ mM) and beef liver ($K_i = 0.23$ mM) in the dark; photolysis at $4.8K_i$ and $3.2K_i$ leads to 48 and 50% covalent inhibitions, respectively.[13] Under the conditions required for photoactivation *etc.* (*e.g.* 5 min at pH 8) the *S*-thioester bond has been found to be hydrolytically-stable. Compound (5) is an N-photolabelled monofunctional analogue of (4), but has not been used in any enzyme systems as yet.

Derivative (6) of GSSG is also bifunctional possessing similar advantages to (4), its GSH bifunctional analogue. It is a very weak substrate of yeast glutathione reductase [k_{cat}/K_m for (6) being *ca.* 10^3-fold smaller than for GSSG]. The K_m value for (6) was 2.07 mM which is close to the value of K_i measured for it when used as a competitive inhibitor of the GSSG-reducing action of glutathione reductase.[14] On photoactivation at $3K_i$ with this enzyme, the GSSG reductase activity of the enzyme is covalently inhibited by *ca.* 30%, but the transhydrogenase activity appears unaffected.[14]

In summary, we have described the synthesis and purification of a series of photolabels based on the GSH–GSSG structure. From the examples above in enzymology, there is a wide potential applicability of these to label covalently GSH-GSSG binding sites in biomaterials, to photolabel transport processes, to cross-link neighbouring sites *etc.* The use of bifunctional photolabels in addition to the obvious cross-linking applications also should increase the specificity of photolabelling. Additional labels are, in principle, available by reductive cleavage of compound (6) or hydrolysis of compound (4); this would produce N-labelled, SH-free GSH derivatives.

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