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Synthesis and evaluation of an N-acylated photoactivatable analogue of glutathione as probe for glutathione-utilizing enzymes

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Abstract—The first synthesis of an N-acylated photoactivatable analogue of reduced glutathione is described. *N*-(4-Benzoylbenzoyl)glutathione (**8**) was found to be an inhibitor and a photoaffinity probe of purified rat liver glutathione *S*-transferases.

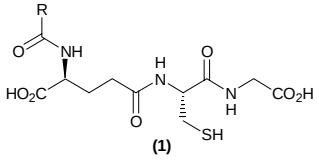
Glutathione in its reduced form (GSH) is the most abundant non-protein thiol in eukaryotic cells. GSH is involved in a variety of important physiological and metabolic functions in all mammalian cells, including detoxification of free radicals, metals and electrophilic xenobiotics such as the lipid peroxidation product 4-hydroxynonenal. The nucleophilic thiol scavenges these compounds, making them more water-soluble and easily eliminated from the cell. This process is mediated by the glutathione *S*-transferase (GST) family of enzymes.[1]

GSH is also known to play an important role in apoptosis.[2a] The apoptotic process[3] is a genetically controlled programmed cell death and is often deficient in cancer cells. A great number of reports on pro- or anti-apoptotic enzymes, which have GSH as substrate, are found in the literature.[1a,4] GSH per se may be essential in the apoptotic process.[2b]

Photoaffinity labelling (PAL) is a powerful technique for the identification of proteins and the characterisation of ligand-binding sites.[5] We are interested in photoactivatable analogues of GSH to probe glutathione-binding proteins. Several studies using photoactivatable glutathione conjugates for the characterisation of GST have been published.[6] However, because of the location of the photoactivatable group on the thiol moiety of GSH, these probes are photoaffinity analogues of glutathione conjugates (GST products) rather than of GSH (GST cosubstrate).

Here, we focussed our attention on N-acylated photoactivatable analogues of GSH (**1**). The originality of these analogues is that their thiol group is free and we hope that their intracellular activity will be close to that of GSH.

To reach these novel photoactivatable analogues of GSH, we first attempted to use the conditions given by Karwatsky et al.[7]



(1)

Indeed, in a recent paper, they described a synthesis of an N-acylated photoactivatable analogue of GSH (**2**) by a direct reaction of an equimolar amount of GSH and *N*-hydroxy-succinimidyl-4-azidosalicylate (**3**) in the presence of 7.5 equivalents of triethylamine (Et_3N) in a mixture of DMF–water as shown in Scheme 1.[7]

N-Hydroxysuccinimidyl-4-azidosalicylate[8] (**3**) (NHS-AS), *N*-hydroxysuccinimidyl-4-azido-benzoate[9] (**4**) (NHS-AB) and *N*-hydroxy-succinimidyl-4-benzoylbenzoate[10] (**5**) (NHS-BB) were prepared according to literature procedures and were allowed to react with GSH in the presence of Et_3N for 48 h (Scheme 2).

In no case studied were we able to observe the presence of the N-acylated GSH analogue. Inspection of the ^1H

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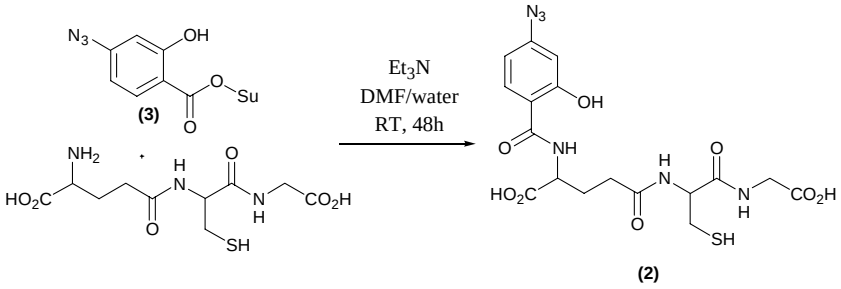
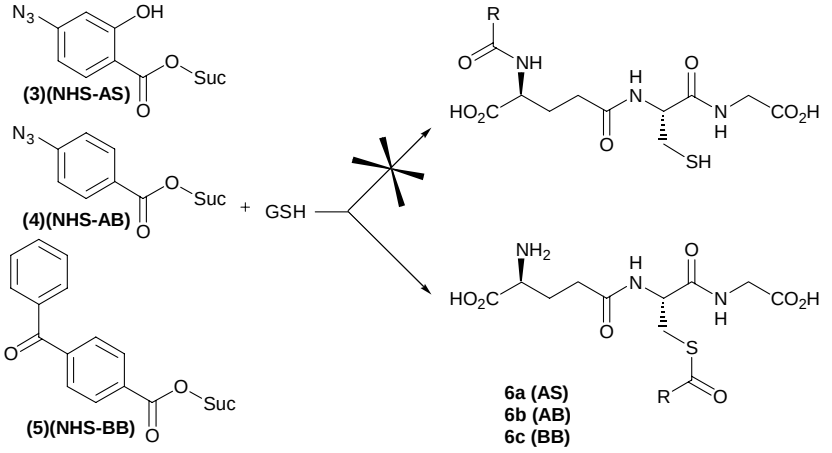


Scheme 1. Karwatsky's synthesis.



Scheme 2. Results using Karwatsky's synthesis.

NMR spectra showed that there was the signal of one single proton at 340–350 ppm in D_2O.[11a] However, the methylene protons of cysteine residue (Cysβ) should have a signal close to 294 ppm counting for 2 protons as GSH in D_2O.[11b,c] Owing to compounds' decomposition at an appreciable rate at room temperature in D_2O, no satisfactory ^{13}C NMR spectra could be recorded despite several attempts. GSH thioesters are known to hydrolyse rapidly in aqueous solution.[12] Moreover, a ninhydrin test revealed the presence of a free amino group and a test using Ellmann's reagent confirmed the lack of free thiol. Application of Karwatsky's protocol led us to compounds (**6a–c**) which are photoactivatable analogues of glutathione conjugates. It may be emphasized that we have conducted the experiment several times precisely under the reported conditions and found that our results are consistently reproducible. Unfortunately all attempts to reach the desired compounds were unsuccessful.

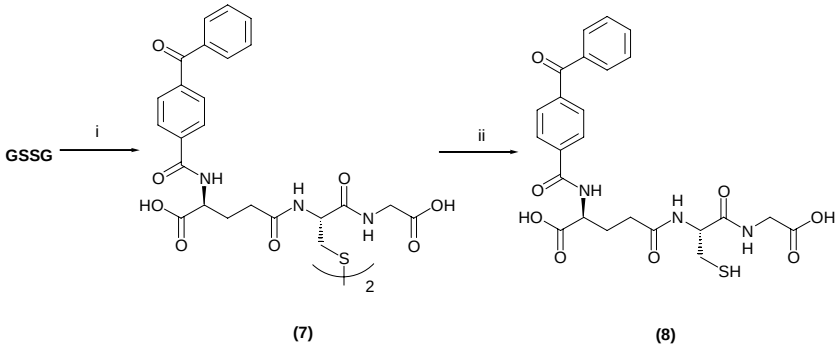
These results are not surprising. Though about 70 protecting groups for thiol function have hitherto been described, the problem of cysteine protection has still remained unsolved.[13] Indeed, the synthesis of peptides containing a cysteine moiety is difficult because the thiol group is more nucleophilic than the amine in the free amino acid.[14]

Moreover, GSH is known to be a good nucleophile and the reactivity of its sulfhydryl group in neutral and basic media is well documented. The thiol function of GSH is involved in nucleophilic substitution,[15] in aromatic nucleophilic substitution,[1a,16] in Michael addition,[17] in epoxide ring opening[18] and in the formation of thioester with activated acids.[19]

The major problem encountered in synthetic reactions for the NH_2 group of S-blocked GSH derivatives is the rather unreactive nature of this group.[20] D'Silva et al. reported that acylation of NH_2 with an acyl chloride must be catalysed by an equimolar amount of *N*,*N*-dimethylaminopyridine (DMAP) to obtain acceptable yields.[21]

Since the direct reaction of GSH with activated esters failed to give the N-acylated analogues of GSH, we proposed to reach *N*-(4-benzoylbenzoyl)glutathione (**8**)[22] in a two-step procedure from glutathione disulfide (GSSG). First, GSSG was N-acylated and then, the disulfide bond of *N*,*N*′-bis-(4-benzoyl-benzoyl)glutathione disulfide[23] (**7**) was reduced with dithiothreitol (dtt) (Scheme 3). Pure (**8**) was obtained in 73% yield from GSSG. Aqueous solution of (**8**) stored at −80 °C is stable for two months before apparition of the corresponding disulfide (**7**) as traces. A similar observation was reported by Gan and co-workers who noticed the oxidation of N-dansylated GSH to *N*,N′-bis-dansylated GSSG.[24,25]

N-(4-Benzoylbenzoyl)glutathione (**8**) was evaluated as an inhibitor of purified rat liver GST used as a model of glutathione-binding protein. The enzyme activity



Scheme 3. Reagents and conditions: (i) **5**, (2 equiv), Et_3N (6 equiv), H_2O, MeCN, rt, 36 h (ii) argon, dtt (5 equiv), H_2O, rt, overnight.

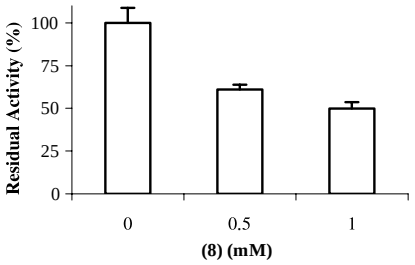


Figure 1. Inhibition of purified rat liver glutathione *S*-transferases by **8**.

was evaluated with 1-chloro-2,4-dinitrobenzene as substrate.[26] A concentration-dependent inhibition of the activity was observed in the presence of (**8**) and was characterised by an IC_{50} (concentration of (**8**) required to produce 50% of inhibition) of about 1 mM (Fig. 1).[27]

We next studied the ability of (**8**) to behave as a GST photoaffinity probe.[27] Figure 2 shows a time-dependent inhibition of the activity observed under UV irradiation in the presence of (**8**). The enzyme inactivation was reduced in the presence of GSH, suggesting a binding of the photoaffinity probe in the GSH-binding site. We verified that the inactivation was dependent on the simultaneous presence of (**8**) and UV since UV alone or (**8**) alone failed to inhibit the GST.

In conclusion, we described here the first N-acylated photoactivatable analogue of GSH in a two-step procedure from GSSG and provided evidence that this compound is a photoaffinity label of glutathione

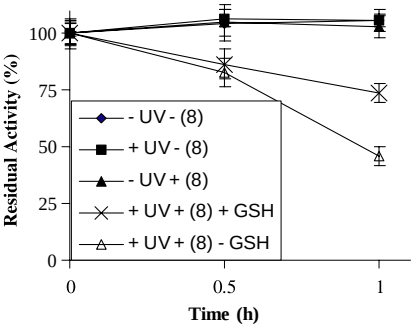


Figure 2. Photoaffinity labelling of purified rat liver glutathione *S*-transferases by **8**.

S-transferases. This new probe should be a valuable tool, which will facilitate the identification and characterization of glutathione-binding proteins and possibly help in deciphering the contribution of the GSH system to apoptosis and drug resistance.

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11. (a) *S-(4-Azido-2-hydroxybenzoyl)glutathione* (***6a***). ^1H NMR (250 MHz, D_2O): $\delta = 8.03–7.99$ (d, $J = 10$ Hz, 1H), 6.82–6.75 (m, 2H), 3.83–3.77 (m, 4H, Gluα, Gly, Cysβ$_1$), *3.48* (m, 1H, Cysβ$_2$), 2.52 (m, 2H, Gluγ), 2.16 (m, 2H, Gluβ); MS (negative ESI): $m/z = 467$ $[M-H]^-$. *S-(4-Azidobenzoyl)glutathione* (***6b***). ^1H NMR (250 MHz, D_2O): $\delta = 8.07–8.04$ (d, $J = 7.5$ Hz, 2H), 7.28–7.25 (d, $J = 7.5$ Hz, 2H), 3.82 (m, 4H, Gluα, Gly, Cysβ$_1$), *3.49* (m, 1H, Cysβ$_2$), 2.58 (m, 2H, Gluγ), 2.22 (m, 2H, Gluβ); MS

(negative ESI): $m/z = 451[M-H]^-$. *S-(4-Benzoylbenzoyl)-glutathione (**6c**)*. ^{1}H NMR (250 MHz, D_2O): $\delta = 8.17$ (d, $J = 8$ Hz, 2H), 8.00–7.90 (m, 5H), 7.68 (m, 2H), 3.86 (m, 4H, Gluα, Gly, Cysβ$_1$), *3.50* (m, 1H, Cysβ$_2$), 2.58 (m, 2H, Gluγ), 2.22 (m, 2H, Gluβ); MS (negative ESI): $m/z = 514[M-H]^-$; (b) Papadia, P.; Margiotta, N.; Bergamo, A.; Sava, G.; Natile, G. *J. Med. Chem.* **2005**, *48*, 3364; (c) Lyon, R. P.; Atkins, W. M. *J. Am. Chem. Soc.* **2001**, *123*, 4408.

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22. *Experimental procedure*. Under argon, dithiothreitol (5 equiv, 22.5 mg, 0.146 mmol) was added at rt to *N*,*N*′-bis-(4-benzoyl-benzoyl)glutathione disulfide (**7**) (30 mg, 0.029 mmol) in distilled water (600 μL). The reaction mixture was then stirred overnight and concentrated under reduced pressure. The crude residue was chromatographed on silica gel column, with a gradient of MeCN–H_2O as eluent.
*N-(4-Benzoylbenzoyl)glutathione (**8**)*. Yield = 86%; ^{1}H NMR (250 MHz, D_2O): $\delta = 7.97$–7.86 (m, 6H), 7.80 (d, $J = 9.2$ Hz, 1H), 7.67–7.64 (t, $J = 9.2$ Hz, 2H), 4.58 (t, $J = 6.9$ Hz, 1H, Cysα), 4.50 (m, 1H, Gluα), 3.77 (s, 2H, Gly), 2.94–2.92 (d, $J = 8.3$ Hz, 2H, Cysβ), 2.59 (m, 2H, Gluγ), 2.20 (m, 1H, Gluβ$_1$), 2.10 (m, 1H, Gluβ$_2$); ^{13}C NMR (62.5 MHz, D_2O): $\delta = 200.03$, 175.69, 171.72, 169.34, 139.71, 137.33, 136.33, 133.83, 130.40, 130.37, 128.60, 127.32, 55.42, 55.04, 42.72, 32.15, 27.01, 25.61; MS (MALDI-TOF): $m/z = 516$ $[M+H]^+$, 538 $[M+Na]^+$; UV/Vis (NaOH 0.05 M): λ_{max} (ε) = 264.5 nm (34,580); Anal. Calcd for $C_{24}H_{25}N_3O_8S$: C, 55.92; H, 4.89; N, 8.15. Found C, 55.84; H, 4.77; N, 8.21.
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26. All reagents were purchased from Sigma. Purified rat liver GST (3.12 μg protein/mL) was assayed at 30 °C in the presence of 1-chloro-2,4-dinitrobenzene (4 mM) and GSH (3 mM) for 4 min, together with (**8**) at the concentrations indicated in the figure. The residual activity was calculated, with the enzyme activity in the absence of (**8**) representing 100% residual activity.
27. Purified rat liver GST (2.96 mg protein/mL) was placed into 1.5 mL microtubes and incubated during 0–1 h at 4 °C under a 365-nm Spectroline UV lamp in potassium phosphate buffer 0.1 M (pH 6.5) in the presence of (**8**) (2 mM). Protein samples were withdrawn at different irradiation times (0–1 h), diluted 1000 times in potassium phosphate buffer 0.1 M (pH 6.5) and assayed for GST activity as described above. The effects of UV irradiation alone and (**8**) alone on the enzymatic activity were evaluated by performing in parallel at 4 °C the irradiation in the absence of (**8**) or the incubation in the presence of (**8**) but without irradiation, respectively. The residual activity (%) was then calculated, with the enzyme activity in the non-irradiated assay at t_0 representing 100% residual activity. GST activity protection against photoinactivation by (**8**) (2 mM) was studied in the presence of GSH (5 mM).