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# Site-selective and covalent labelling of the cysteine-containing peptide glutathione with a ferrocenyl group

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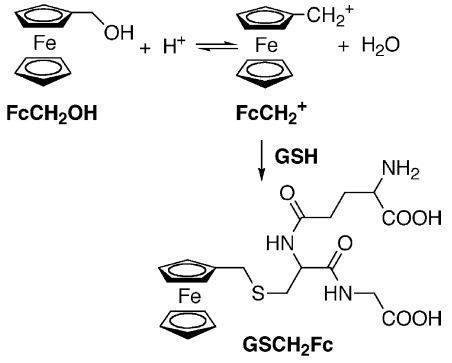
**Abstract**—Site-specific labelling of the cysteine-containing peptide glutathione with a ferrocene group was achieved by reaction with ferrocenylmethanol in aqueous acidic medium. The resulting peptide was shown to be a potent competitive inhibitor of the biologically important enzyme glutathione-(*S*)-transferase. This approach may prove general for the labelling of proteins with ferrocene. Published by Elsevier Ltd.

Several synthetic methods aimed at the selective conjugation of organo-iron species (including the electro-active ferrocenyl entity Fc) to peptides and proteins are now available from the literature. All these methods take advantage of the reaction of particular organo-iron complexes with nucleophilic functions present on the N-terminal amino acid,[1] or on the side chain of certain amino acids, such as the ε-amino group of lysines or the β-sulfhydryl group of cysteines or even the imidazole group of histidines.[2]

To tag the β-sulfhydryl group of cysteine residues, the first straightforward approach consisted in its reaction with Fc and Fp ($CpFe(CO)_2$) derivatives designed after classical protein chemistry reagents.[3–8] Another approach took advantage of the intrinsically electrophilic cation $[(\eta^5\text{-R-}C_6H_6)Fe(CO)_3]^+$ (R = H, 2-OMe). [9] However side reactions with other nucleophiles present in the reaction medium may occur. A smarter labelling route involving spontaneous in situ generation of the above-mentioned cation was taken by Stephenson and colleagues to label enzymes with ($\eta^4$-cyclohexadiene)tricarbonyl iron(0) moieties.[10]

It has been previously shown that *N*-acetylcysteine reacts with ferrocenylmethanol **$FcCH_2OH$** in THF in the presence of a catalytic amount of TFA to yield the thioether derivative.[11] This reaction took advantage of the facile in situ generation of the highly stabilized α-ferrocenylcarbenium ion in the presence of a strong acid and its subsequent addition to the thiol. In the present article, we describe a new general approach to introduce an Fc group into cysteine-containing peptides and proteins based on the reaction of **$FcCH_2OH$** in aqueous medium. As an example, the reaction of the natural tripeptide glutathione (γ-glutamylcysteinylglycine, **GSH**) with **$FcCH_2OH$** in a water/acetone mixture in the presence of a catalytic amount of TFA led after workup to an orange solid. The $^1$H NMR spectrum of this product recorded in DMSO-$d_6$ was fully consistent with the structure of **$GSCH_2Fc$** depicted in Scheme 1, resulting from the attachment of the $FcCH_2$ unit to the cysteine




**Scheme 1.** Mechanism of formation of **$GSCH_2Fc$**.

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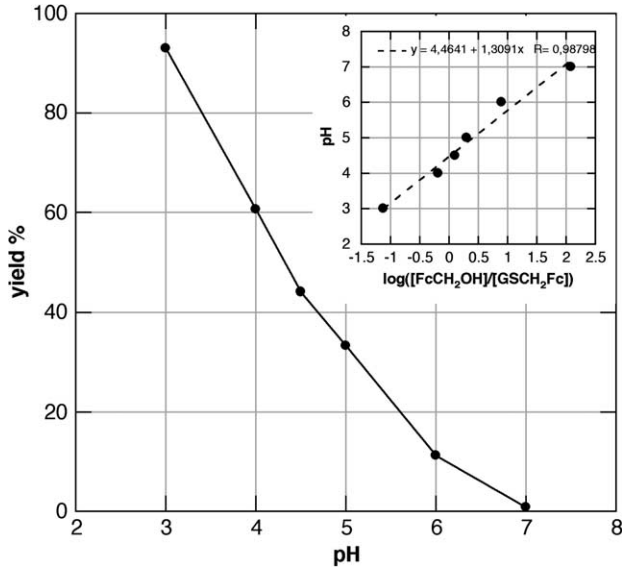


**Figure 1.** Yield in **$GSCH_2Fc$** as a function of pH. Solvent: 0.1 M citrate–phosphate buffer/acetone 10/1 (v/v). Inset: measurement of $K_{app}$.

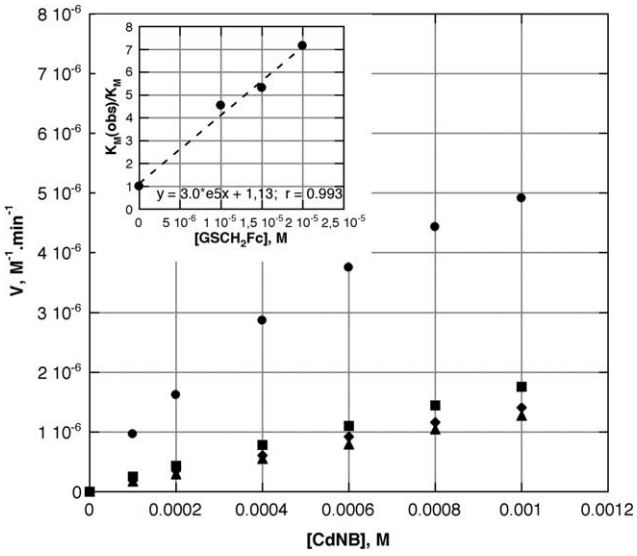


**Figure 2.** Competitive inhibition of **GST** by **$GSCH_2Fc$** with **CdNB** as variable substrate. [**GSH**] = 0.2 mM. (●) [**$GSCH_2Fc$**] = 0; (■) [**$GSCH_2Fc$**] = 10 μM; (◆) [**$GSCH_2Fc$**] = 15 μM; (▲) [**$GSCH_2Fc$**] = 20 μM. Inset: Dixon plot.

sulfur. **$GSCH_2Fc$** was also characterized by IR and high resolution mass spectroscopy.[12]

Analytical reverse-phase HPLC of mixtures of equimolar amounts of **$FcCH_2OH$** and **GSH** in buffered aqueous solutions after 48 h incubation time revealed that **$GSCH_2Fc$** was the only product formed (i.e., no reaction takes place with the α-amine of the glutamic acid residue).[13] The yield of formation of **$GSCH_2Fc$** calculated from the **$FcCH_2OH$** and **$GSCH_2Fc$** peaks' area appeared to be markedly dependent on the pH of the reaction medium (Fig. 1). Formation of **$GSCH_2Fc$** was almost quantitative a pH 3.0 and decreased gradually until the pH reached 7. This finding is in agreement with the mechanism depicted above involving the intermediate formation of the α-ferrocenylcarbenium ion **$FcCH_2^+$**. An apparent equilibrium constant $K_{app}$ = [**$GSCH_2Fc$**]/[**$FcCH_2OH$**] × [$H^+$] of $10^{4.5}$ was calculated (Fig. 1, inset). By comparison, the previously reported constant of the carbinol/carbenium equilibrium $K_{R+}$ = [**$FcCH_2^+$**]/[**$FcCH_2OH$**] × [$H^+$] was equal to $10^{-1.17}$,[14] confirming the high thiophilicity of the α-ferrocenylcarbenium intermediate.[15]

Finally, the effect of **$GSCH_2Fc$** on the conjugation of 1-chloro-2,4-dinitrobenzene (**CdNB**) to **GSH** catalyzed by the enzyme glutathione-(*S*)-transferase (**GST**) from equine liver was studied.[16] This initial velocity $V$ of the reaction of GSH (0.2 mM) was plotted as a function of the concentration of **CdNB** for three different concentrations of **$GSCH_2Fc$** (Fig. 2). It was observed that the enzymatic catalysis of the conjugation reaction followed a Michaelis–Menten process and that **$GSCH_2Fc$** behaved as competitive inhibitor of **GST** with an inhibition constant $K_i$ of 3.8 ± 1.4 μM as calculated from the Dixon plot (Fig. 2, inset). It appeared that **$GSCH_2Fc$** is a much better inhibitor of **GST** than our previously described organometallic derivative of **GSH** (*S*)-[3-Fp-($\eta^1$-*N*-succinimidato)]glutathione for which an inhibition constant of 35 μM had been measured.[17] **$GSCH_2Fc$** was also roughly a two times less efficient inhibitor than the powerful competitive inhibitor (*S*)-hexylglutathione for which we have measured a $K_i$ of 2 μM.

In conclusion, we have shown that the site-specific labelling of the cysteine-containing peptide glutathione with a ferrocenyl unit could be achieved in high yield by reaction of ferrocenylmethanol in acidic aqueous solution. The effect of the pH on the reaction yield was explained by that the mechanism of reaction involving the formation of an intermediate α-ferrocenyl carbenium ion. The resulting ferrocenyl peptide was finally shown to behave as a potent competitive inhibitor of the biologically important enzyme glutathione-(*S*)-transferase.

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12. General procedure: In a Schlenk tube under argon, **$FcCH_2OH$** (0.432 g; 2 mmol) and **GSH** (0.614 g; 2 mmol) were dissolved in a 1/1 acetone/water mixture (13 mL) and three drops of TFA were added. The mixture was heated to 45–50 °C and stirred for 2 h then left to stand overnight at room temperature. The orange precipitate of **$GSCH_2Fc$** was filtered off, washed with water and acetone and dried in the air (yield 86%). $^1$H NMR (∂, 300 MHz, DMSO-$d_6$) 1.9 (br, 2H, C$H_2$–CH) 2.3 (br, 2H, $CH_2$–CO) 2.55 (m partially masked by DMSO, $CH_2$–S) 2.82 (dd, 5 and 12 Hz, 1H, $CH_2$–S) 3.30 (br, 1H, CH of Cys) 3.51 (s, 2H, C$H_2$-Cp) 3.7 (br, C$H_2$–COOH) 4.08 (t, 1.7 Hz, 2H, Cp-$H_{3,4}$) 4.12 (s, 5H, Cp′) 4.19 and 4.21 (d, 1.7 Hz, 1H, Cp-$H_{2,5}$) 4.45 (m, 1H, C*H*–COOH of Glu) 8.3 (d, 10 Hz, 1H, NH of Cys) 8.6 (m, 1H, NH of Glu). IR (KBr) 3447, 3376 (NH, OH) 3079 (CH of Cp) 2954 (CH) 1670 (C=O acid) 1651 (C=O amide). HR-MS 506 [$MH^+$] Calcd for $C_{21}H_{28}N_3O_6SFe$ 506.10483. Found 506.10207.
13. Analytical conditions: System GOLD (Beckman Coulter) equipped with a pump 126 and a diode-array detector 168. Samples (20 μL) were injected into a Kromasil C8 (5 μm, 4.6 × 250 mm) column (Eka Nobel) equilibrated with 10% of a 0.1% TFA/MeCN mixture in 0.1% TFA/$H_2O$. Species were eluted at a flow rate of 1 mL/min by application after 5 min of a linear gradient to 100% in 20 min. Absorbance was monitored at 230 nm. Retention time of **$GSCH_2Fc$** and **$FcCH_2OH$**: 16.5 and 19.5 min, respectively.
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16. Enzymatic assay: Absorption at 340 nm of 1 mL-solutions containing **GST** (2 μg/mL), **GSH** (0.2 mM) and **CdNB** in 0.1 M phosphate buffer containing 1 mM EDTA was monitored for 10 min at 22 ± 0.5 °C, in absence or presence of a variable concentration of **GSFc** or (*S*)-hexylglutathione.
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