Bio-orthogonal chemistry methodologies and chemical biology of cysteine redox signalling

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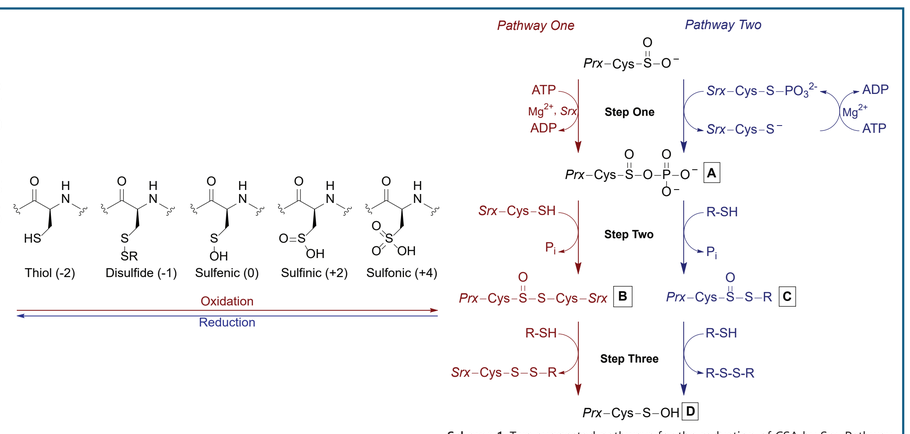
Introduction

Cysteine exists in a variety of oxidation states ranging from -2 to +4, owing to sulfur's ability to expand its octet. We are interested in the +2 oxidation state of cysteine - cysteine sulfinic acid (CSA). CSA was thought to be an irreversible overoxidation of cysteine until 2003, when Woo *et al.*[1] found through metabolic labelling that CSA could be converted to the corresponding thiol form *in vivo* through an unknown mechanism. Sulfiredoxin (Srx), the protein that reduces CSA in peroxiredoxins (Prx), was discovered by Biteau *et al.*[2] later in 2003 and the mechanism of its reduction was confirmed by Jonsson *et al.*[3, 4] using various crystal structures. Chemical proteomics studies by Atker et al.[5] in 2018 revealed ~55 new targets for Srx, not related to the Prxs, opening up the potential functions of CSA in the body as a reversible protein marker.

Our aims and objectives are:
- To gain a greater understanding of the chemical properties of CSA.
- Eluctidate the molecular recognition and specificity of Srx.
- To create a method that allows for the introduction of CSA into a variety of compounds using a protecting group which would be stable to various conditions and only be cleaved upon expusure to nucleophilic deprotection conditions.
- Investigate the intrinsic chemical stability of the CSA functional group using NMR studies as a function of pH and temperature.
- Develop a method to incorporate CSA into peptides in order to build a library of CSA containing peptides.



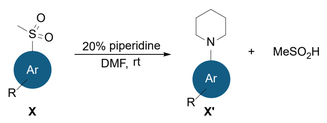
Scheme 1. Two suggested pathways for the reduction of CSA by Srx: Pathway one presented by Biteau *et al.*,[2] Pathway two presented by Jeong *et al.*,[6] Pathway one proven correct by Jonsson *t al.*,[3, 4] scheme adapted from [3].

Protecting Group Development

A tuneable protecting group (PG) was developed to facilitate the selective synthesis of sulfinic acid derivatives. Depending on the final compound required, different substituents on the PG could be used to allow for ease of deprotection/ stability to a range of conditions and only fall under a specific set of nucleophilic deprotection conditions we have developed.

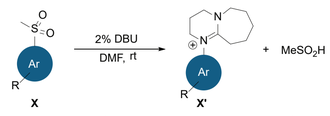
To test which PG substituents worked best, a series of compatibility testing reactions were carried out. These tests used a model methylsulfonyl substituted system, designed to mimic the reactivity of other sulfonyl derivatives. Due to our interest in SPPS, Fmoc deprotection conditions were first trialled - 20% piperidine in DMF, then 2% DBU in DMF (a milder alternative to piperidine).

Results showed that 5-position EDG were the best option for stability all round, however these were lower yielding synthetic reactions, due to the EDGs deactivating nature. Tests with DBU provided an additional two options, 4 or 4,6-methyl substituents. For synthetic routes not requiring a basic step, the 4,6-dimethoxy substituent was shown as an additional option and is commercially available.



Entry	Substituent (R)	Compound (X)	Conversion after 2 hours	Conversion after 24 hours
1	5-methyl ester	1	100%	-
2	5-methoxy	2	0%	0%
3	4/5/6-H	3	~90%	100%
4	4-methoxy	4	100%	-
5	4,6-dimethoxy	5	100%	-
6	4-methyl	6	~80%	100%
7	5-methyl	7	~40%	~80%
8	4,6-dimethyl	8	~60%	~90%
9	5-chloro	9	100%	-
10	5-bromo	10	100%	-
11	5-iodo	11	100%	-
12	5-fluoro	12	100%*	-
13	4-chloro	13	~90%*	100%*
14	5-(1-piperidinyl)	14	0%	0%
15	4-(1-piperidinyl)	15	~20%	~40%
16	4-(diethylamino)	16	~10%	~30%

Table 1. Compatibility testing results – the emergence of the piperidinyl derivative, the unwanted SNAr product X', was monitored by LCMS. Percentage conversion estimated based upon the relative peak integration from the UV absorbance (254 nm) chromatogram by LCMS (assuming consistent solubility and UV absorbance); full conversion equals complete consumption of starting material (*an alternative 4- or 5- substituted product was synthesised).



Entry	Substituent (R)	Compound (X)	Conversion after 2 hours	Conversion after 24 hours
1	4,6-dimethoxy	5	5%	50%
2	4,6-dimethyl	8	0%	0%
3	5-methoxy	2	0%	0%
4	4/5/6-H	3	0%	5%
5	4-methyl	6	0%	0%
6	4-methoxy	4	0%	5%

Table 2. Compatibility testing results – the emergence of the DBU substituted derivative, the unwanted SNAr product X', was monitored by LCMS. Percentage conversion estimated based upon the relative peak integration from the UV absorbance (254 nm) chromatogram by LCMS (assuming consistent solubility and UV absorbance).

NMR Stability Studies

Using NMR, we have investigated the stability of the CSA functional group in a range of buffers at different temperatures.
An NMR method with water suppression allowed the visualisation of the peaks of interest, NMRs were run at 24h intervals at pH 4, pH 7 and pH 9. Initial studies were run at 25°C, where data showed no difference in peak chemical shift or integration over a period of 7 days. This then led to further investigations at 37°C (to allow comparision of in vitro vs in vivo stability) and also 60°C to determine the stability at extremes.
The results of these studies again showed no change in chemical shift or integration suggesting that the CSA functionality is generally stable even at higher temperatures.

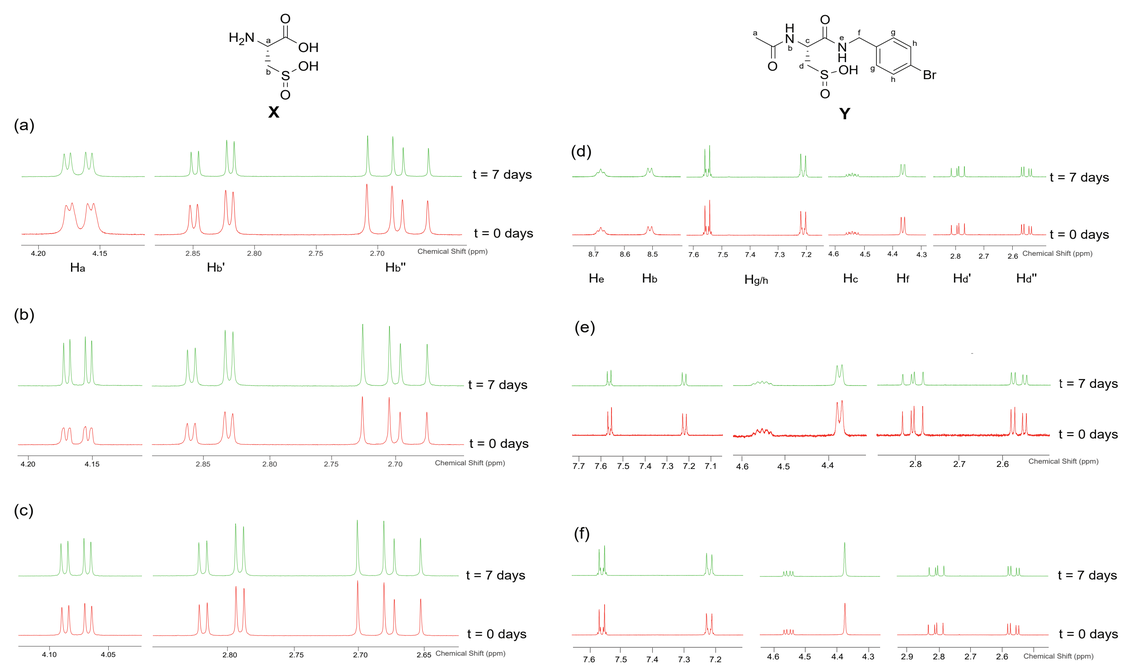
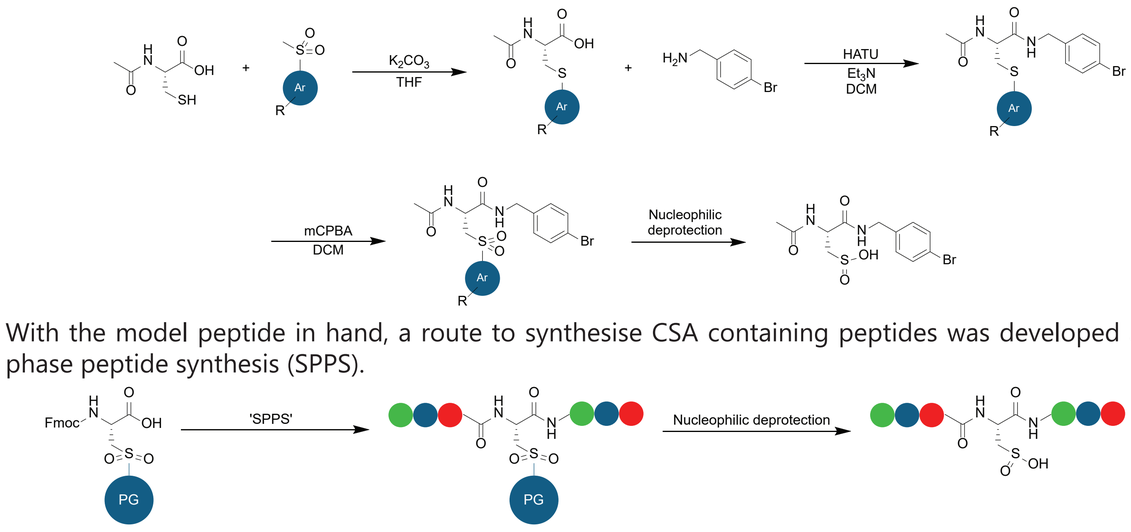


Figure 1. (a) CSA (X), pH 4, 25°C (b) X, pH 7, 25°C (c) X, pH 9, 25°C (d) model CSA containing peptide (Y), pH 4, 25°C (e) Y, pH 7, 25°C (f) Y, pH 9, 25°C

Synthesis of CSA containing compounds

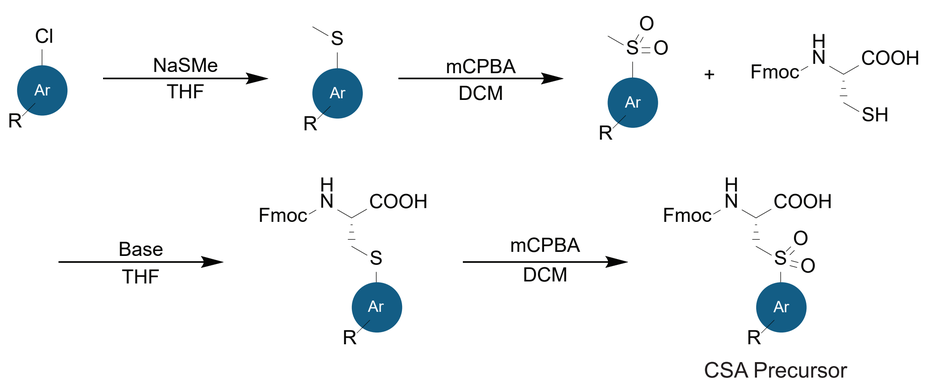
With a method of protecting the CSA functionality in hand, different ways to incorporate CSA were investigated. We initially needed a CSA containing model peptide system to be able to be used in NMR stability studies.
Two different iterations of the model peptide were synthesised but unfortunately the first was far too polar to successfully separate from any impurities. Therefore, we added a bulky hydrophobic phenyl ring to decrease the polarity of the system. This CSA incorporated peptide was synthesised in 4 steps as shown in the figure below.



With the model peptide in hand, a route to synthesise CSA containing peptides was developed using solid phase peptide synthesis (SPPS).

An Fmoc protected, sulfinic acid protected cysteine derivative (CSA precursor) was required for SPPS to be successful. Using the most stable protecting group found during compatibility testing, the CSA precursor was designed and then synthesised.

Most of the synthesis was straight forward, however, S_NAr using Fmoc protected cysteine proved difficult. This was due to the slow rate of reaction caused by the highly deactivating 5-postition EDG. A series of different bases, timings and conditions were trialled until one was found to give the highest yield and the lowest amount of Fmoc deprotection (an issue found with some bases due to its base lability).



With the CSA precursor in hand, the novel amino acid is ready to be incorporated into peptides using SPPS, then deprotected prior to global deprotection using an additional nucleophilic deprotection step.

Conclusions and Future Work

A protecting group to enable the selective incorporation of cysteine sulfinic acid was developed and CSA containing compounds were synthesised. Using one of these compounds and CSA, a series of stability tests were carried out showing that CSA is stable in various buffers at different temperatures for prolonged periods of time.
In the near future, a library of peptides will be synthesised which incorporate CSA. These peptides will have a range of sequences and CSA placed at different positions potentially allowing for further investigation into the selectivity and role of Srx.
Future studies using CSA derivatives could help give a greater understanding into the role of CSA in proteins and redox regulation. This new understanding may lead to applications in diagnostics and drug discovery for as yet unknown disease pathways.

References

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