

One-Pot Thiol-Amine Bioconjugation to Maleimides; Simultaneous Stabilisation and Dual Functionalisation

Archie Wall, alfie wills, Nafsika Forte, Calise Bahou, lisa bonin, Nicholls Karl, michelle ma, Vijay Chudasama, James Baker

Submitted date: 11/06/2020 • Posted date: 12/06/2020

Licence: CC BY-NC-ND 4.0

Citation information: Wall, Archie; wills, alfie; Forte, Nafsika; Bahou, Calise; bonin, lisa; Karl, Nicholls; et al. (2020): One-Pot Thiol-Amine Bioconjugation to Maleimides; Simultaneous Stabilisation and Dual Functionalisation. ChemRxiv. Preprint. <https://doi.org/10.26434/chemrxiv.12465176.v1>

Maleimide chemistry is widely used in the site-selective modification of proteins. However, hydrolysis of the resultant thiosuccinimides is required to provide robust stability to the bioconjugates. Herein, we present an alternative approach that affords simultaneous stabilisation and dual functionalisation in a one pot fashion. By consecutive conjugation of a thiol and an amine to dibromomaleimides, we show that aminothiomaleimides can be generated extremely efficiently. Furthermore, the amine serves to deactivate the electrophilicity of the maleimide, precluding further reactivity and hence generating stable conjugates. We have applied this conjugation strategy to peptides and proteins to generate stabilised trifunctional conjugates. We propose that this stabilisation-dual modification strategy could have widespread use in the generation of diverse conjugates.

File list (2)

Amine-Thiol Dual Conjugation Manuscript.pdf (1.38 MiB)

[view on ChemRxiv](#) • [download file](#)

Amine-Thiol Dual Conjugation ESI.pdf (27.50 MiB)

[view on ChemRxiv](#) • [download file](#)

One-Pot Thiol-Amine Bioconjugation to Maleimides; Simultaneous Stabilisation and Dual Functionalisation

Archie Wall,[†] Alfie G. Wills,[†] Nafsika Forte,[†] Calise Bahou,[†] Lisa Bonin,[†] Karl Nicholls,[‡] Michelle T. Ma,[§] Vijay Chudasama^{†,¶,*} and James R. Baker^{†,*}

[†] Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK

[‡] Albumedix Ltd, Mabel Street, Nottingham, NG2 3ED, UK

[§] School of Biomedical Engineering and Imaging Sciences, King's College London, St Thomas' Hospital, London SE1 7EH, UK

[¶] Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal

Abstract

Maleimide chemistry is widely used in the site-selective modification of proteins. However, hydrolysis of the resultant thiosuccinimides is required to provide robust stability to the bioconjugates. Herein, we present an alternative approach that affords simultaneous stabilisation and dual functionalisation in a one pot fashion. By consecutive conjugation of a thiol and an amine to dibromomaleimides, we show that aminothiomaleimides can be generated extremely efficiently. Furthermore, the amine serves to deactivate the electrophilicity of the maleimide, precluding further reactivity and hence generating stable conjugates. We have applied this conjugation strategy to peptides and proteins to generate stabilised trifunctional conjugates. We propose that this stabilisation-dual modification strategy could have widespread use in the generation of diverse conjugates.

Introduction

Maleimides represent one of the most widely used functional groups for conjugation chemistry.^{1–4} This is due to the extremely favourable kinetics of their reaction with thiols, which ensures high yields of the desired conjugates are generated rapidly whilst minimising competing side-reactions. This has led to their use in fields ranging from therapeutic bioconjugates,^{5,6} to multifunctional polymers and a diverse array of materials.^{7,8} Despite this widespread use, maleimides suffer several limitations which have been widely noted. Most significantly, the reaction generates succinimides **1** that are unstable over prolonged time *in vivo*, undergoing retro-conjugate additions and subsequent trapping with endogenous thiols such as glutathione.^{9–12} To overcome this, the succinimides must be hydrolysed post-conjugation, which can lead to a reduction in the yield of the conjugates,^{13,14} prolonged reaction times and undesirable exposure to high pH conditions. Furthermore, the hydrolysis generates two regioisomeric maleamic acids **2a** and **2b**, which when combined with the formation of a stereocenter in the initial conjugate addition, results in four isomeric products (**Fig. 1A**).

To overcome these limitations we, and others, have reported on the use of dibromomaleimides (DBMs),^{15–20} and related substituted maleimides known collectively as next generation maleimides (NGMs).^{19,21} These reagents retain the favourable kinetics of maleimides whilst avoiding the formation of stereoisomers, and through the addition of two thiols allow the construction of dual conjugates (**Fig. 1B**). Whilst this *bis*-thiol conjugation has found widespread use in the bridging (also known as stapling) of disulfide bonds it has found less application to intermolecular conjugates, as the dithiomaleimide products **3** are still thiol reactive and thus the control of stoichiometry is challenging to prevent scrambling of the thioethers. Furthermore, hydrolysis post-conjugation is required to confer *in vivo* stability, which still generates a mixture of two regioisomeric maleamic acids.^{10,11}

We hypothesized that a completely different mechanism for maleimide conjugate stabilization might prove viable, overcoming the limitations of imide hydrolysis whilst offering new opportunities for the construction of multifunctional conjugates. The addition of an amine to a dibromomaleimide-thiol conjugation reaction would afford an aminothiomaleimide **4** (**Fig. 1C**), in which the electron donating amino group would deactivate the maleimide to further nucleophilic attack. The product would thus be stable, precluding the requirement for post-conjugation hydrolysis and generating a

single isomer (**Fig. 1C**). Furthermore, the amine would represent an additional site for functional attachment, thus representing an ideal scaffold for the construction of triconjugates.

It has previously been shown that dibromomaleimides (DBMs) react selectively with thiols over amines, hence the initial addition of thiol was expected to occur rapidly and selectively.^{15,20} Amine addition to dihalomaleimides has been reported to generate aminohalomaleimides, suggesting that whilst the reaction is slower than for thiols it is still viable.²² Interestingly, thiol addition to aminobromomaleimides has been found to occur only under high temperatures (80 °C),²³ indicating that the amino group does indeed dramatically reduce the maleimide's electrophilicity. Whilst promising indicators for the desired reaction sequence, far milder reaction conditions would be required for this reaction to be broadly applicable as a bioconjugation strategy. For protein conjugates the reactions would have to take place at, or near, room temperature, at high dilution and be effective in aqueous buffered conditions in which most amines would be substantially protonated.

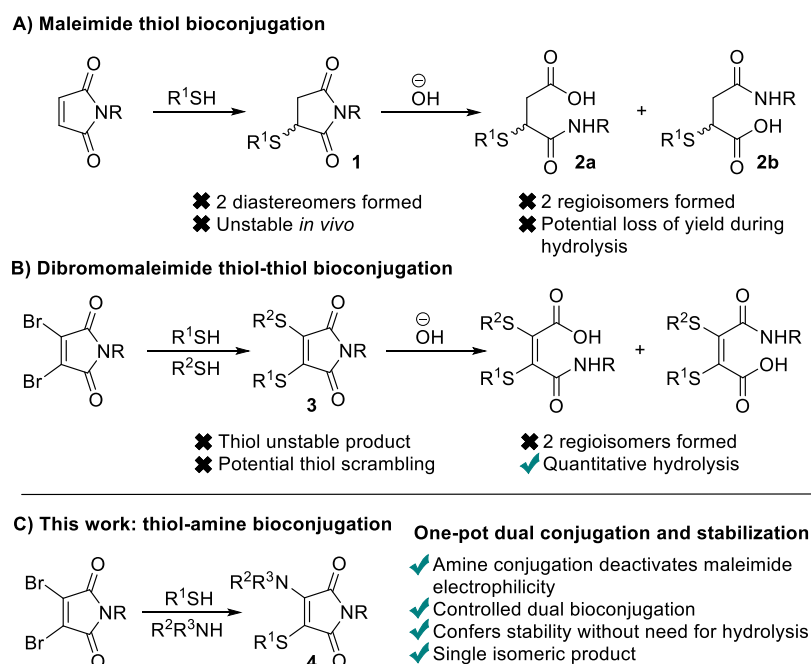


Figure 1: (A) Thiol conjugation with classical maleimides. (B) Dual thiol conjugation using Next Generation Maleimides (NGMs). (C) Dual thiol-amine conjugation with simultaneous stabilisation described herein.

Results and Discussion

Bromothiomaleimides **5** and **6** were synthesized as test substrates for initial tests of amine additions. Enticingly all amines trialled conjugated efficiently in methanol to generate the desired aminothiomaleimides **7-12** in excellent yields (**Fig. 2**), with both

N-aryl and *N*-alkyl substituents. As expected, the more nucleophilic secondary alkyl amines, such as piperidine and pyrrolidine, reacted more efficiently than aryl amines (ca. 10 min vs 24 h, see **ESI Table S1** for more details).

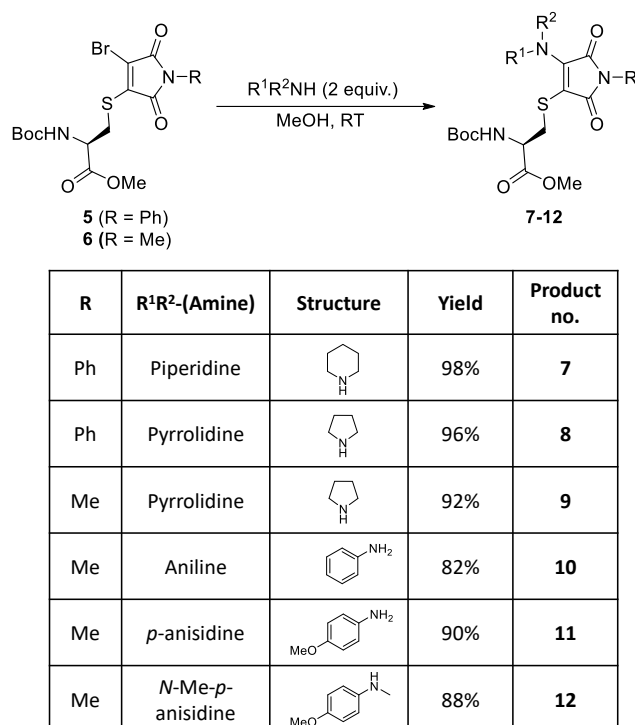


Figure 2: Reactions of bromothiomaleimides **5** and **6** with amines in methanol.

Whilst this study shows the promising potential of this dual conjugation sequence in organic solvent, we moved on to test aqueous buffered conditions. Analysis of the relative rates of amine addition under aqueous conditions (pH 7.8, 4.7 mM, with 2 equiv. of amine) was carried out using NMR, via the integration of the starting material and product *N*-methyl peaks (**Fig. 3**). We observed that aniline ($pK_{\text{aH}} = 4.87$) offered a substantial rate enhancement over pyrrolidine ($pK_{\text{aH}} = 11.31$), confirming that protonation was substantially attenuating reactivity of the secondary amine. The pyrrolidine reaction was also observed to generate undefined side products by crude NMR analysis, and was therefore ruled out as a viable reagent. In contrast, incorporating a *para*-methoxy group onto the aniline, which subtly raises the pK_{aH} (*p*-anisidine, $pK_{\text{aH}} = 5.36$), afforded the most reactive amine under these conditions. *p*-Anisidine represented the best balance between nucleophilicity and pK_{a} for undertaking these reactions at near neutral pH,^{24,25} yielding a single product and reaching completion in 185 min. The aminothiomaleimide **11** was then assessed for stability by prolonged incubation in: pH 7.4 buffer, pH 5 buffer and also 12 mM thiol

solution (2-mercaptoethanol). The resulting NMR data confirmed the product as completely stable in all conditions (monitored for 6-8 days, see **ESI Fig. S23-S26**). The *p*-anisidine conjugation to bromothiomaleimides was thus shown to be an extremely promising new bioconjugation reaction, occurring under mild aqueous conditions and generating stable products.

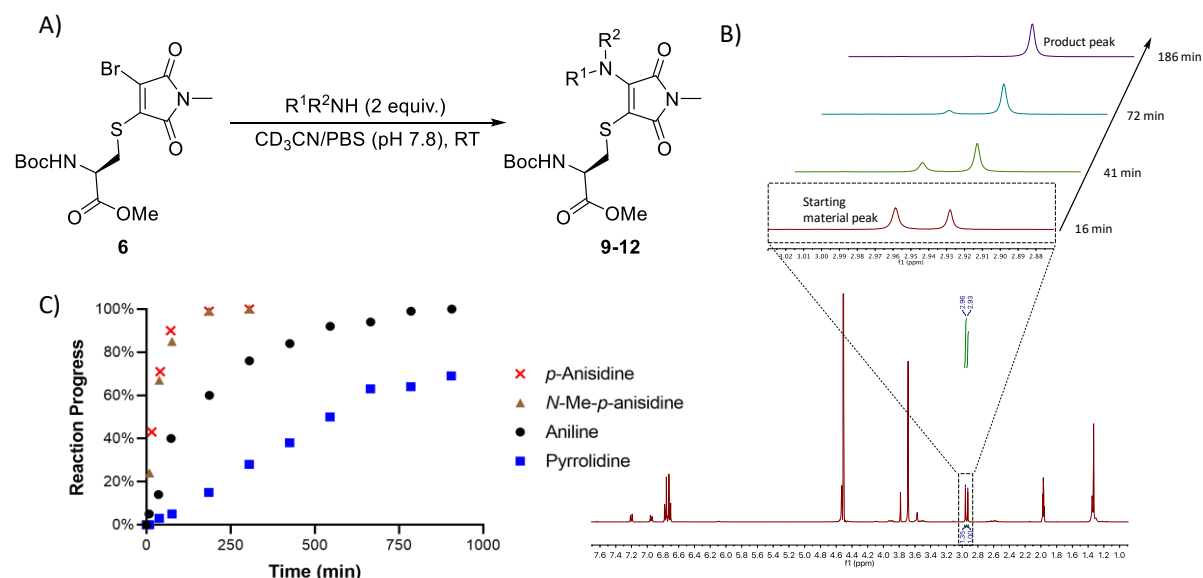


Figure 3: (A) General reaction scheme for NMR time course reactions. (B) Methyl peak development observed in time course 1H NMR study for reaction between *p*-anisidine and bromothiomaleimide **6**. (C) Graph showing the progress of 4 key amine reactions under buffered conditions (see **ESI Table S3**).

To trial this strategy in protein bioconjugations, wild-type human serum albumin (HSA) was chosen as an initial target; it contains a single cysteine (Cys-34) and HSA-conjugates are of clinical interest as a leading platform for half-life extension.^{26–29} A useful feature of this chemistry is the ability to monitor the progress of both thiol and amine additions by UV-vis spectroscopy, as distinct λ_{max} values are associated with the bromothiomaleimide (ϵ_{375} 2910 M^{-1}) and the red-shifted aminothiomaleimide products (ϵ_{415} 4250 M^{-1}). The conjugation of DBM **13** to the protein was complete after just 20 min, confirmed by UV (**Fig. 4**), and subsequently LCMS analysis (**ESI Fig. S7**). *p*-Anisidine (50 mM) was then added to the reaction mixture and the absorbance was monitored at 415 nm, taking 180 min to plateau. LCMS analysis confirmed that the amine addition had taken place (**Fig. 4**). It was unsurprising to observe relatively long reaction times on HSA, with high concentrations of amine required to ensure the reaction went to completion; this is likely due to the buried nature of the Cys-34 cleft, and the fact that a proximal nucleophilic Tyr-84 has been proposed to intercept dihalomaleimide-HSA conjugates, as both halide atoms are lost from the backbone of the DBM during conjugation.¹⁹

To preclude these specific issues associated with wild-type HSA, we shifted our focus onto an HSA mutant (mHSA) with an alternative cysteine location (C34A + K93C), kindly provided by Alumedix.³⁰ It was pleasing to observe in this case that the bromothiomaleimide was present in the LCMS analysis of the intermediate prior to amine addition, indicating that there were no reactions with other nucleophilic residues near the conjugation site (**ESI Fig. S8**). Interestingly, *p*-anisidine addition was rapid (~ 20-fold faster compared to WT HSA, see **Fig. 4**) for mHSA intermediates, suggesting that the amine could be reacted at much lower concentrations. Furthermore, we postulated that the rate difference between thiol and amine additions would enable an *in situ* reaction, in which DBM **13** (3 equiv.) was added to a mixture of the protein and the *p*-anisidine (5 equiv.). This proved effective after being left overnight at RT (**ESI Fig. S13**), demonstrating this thiol-amine dual conjugation to be a rare example of an efficient multi-component reaction on a protein. Finally, conjugate **17** was assessed for thiol stability (glutathione, GSH). Treatment with 10 μ M GSH (representative of extracellular concentrations)^{31–34}, pH 7.4, 37 °C for 7 days, showed stability by LCMS analysis (see **ESI**). A further experiment was carried out to approximate the harsh intracellular GSH concentrations (4 mM GSH, pH 6.8, 37 °C)^{31–34} and showed little change after 24 h (see **ESI**). In summary, *p*-anisidine addition was shown to offer an alternative approach to hydrolysis for maleimide bioconjugate stabilisation. It offers the further advantages of taking place under mild conditions, can be accelerated simply by increasing the amine concentration and can be carried out step-wise or *in-situ*.

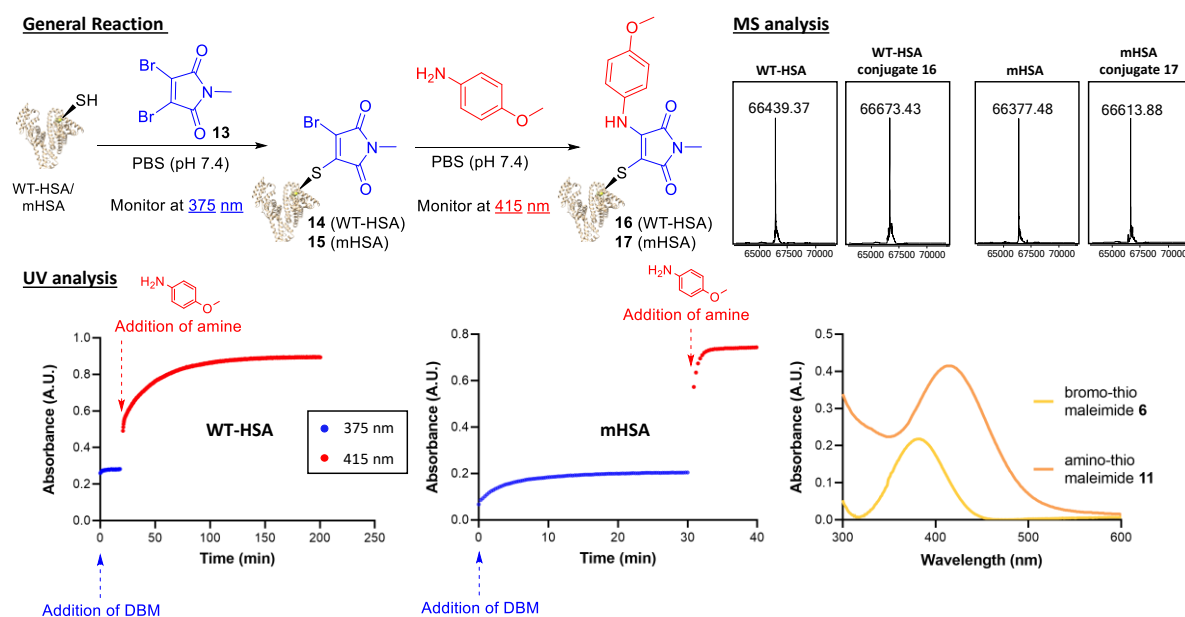


Figure 4: General reaction scheme for WT-HSA and mHSA conjugations (UV monitoring displayed below for each protein). Desired product confirmed by LCMS. Expected mass for conjugate **16**: 66,671 Da. Observed mass: 66,673 Da. Expected mass for conjugate **17**: 66,612 Da. Observed mass: 66,614 Da. See **ESI** for more detail.

A further enticing opportunity offered by this conjugation methodology was the possibility of generating triconjugates, if a heterobifunctional amine based on the *p*-anisidine core was used. To this end, two variants of *p*-anisidine, alkyne **18** and azide **22** were synthesised that would enable construction of clickable triconjugates. These scaffolds would then facilitate further modification as desired through the plethora of well-defined click chemistry currently available, depending on the nuances of both protein and probe in use. Importantly, functional analogues **18** and **22** displayed very similar reaction rates with the bromothiomaleimide **11** as *p*-anisidine (**ESI Fig. S2**).

Our first test of this dual conjugation strategy was trialled on a peptide. Cell penetrating peptides (CPPs) have garnered interest as a means to achieve payload internalisation in a cell.^{35–37} P218R is an optimised cell-penetrating peptide designed by Dixon *et al.* and represents an extremely challenging modification target due to the abundance of lysine and arginine residues present (9 and 11 residues respectively) in a peptide of 3.8 kDa.³⁸ Interestingly, the rapid kinetics of the thiol conjugation enabled efficient and rapid dual modification of the CPP via an *in situ* protocol (40 equiv. amine-alkyne **18**, 1.2 equiv. DBM **19**, 30 min at 37 °C, see **Fig. 5**), indicated by crude LCMS data (**ESI Fig. S20**) and immediate colour change upon addition of DBM **19**.

Subsequent HPLC purification afforded conjugate **20** (**Fig. 5**). Work with modified analogues of **20** is underway for use as imaging agents.

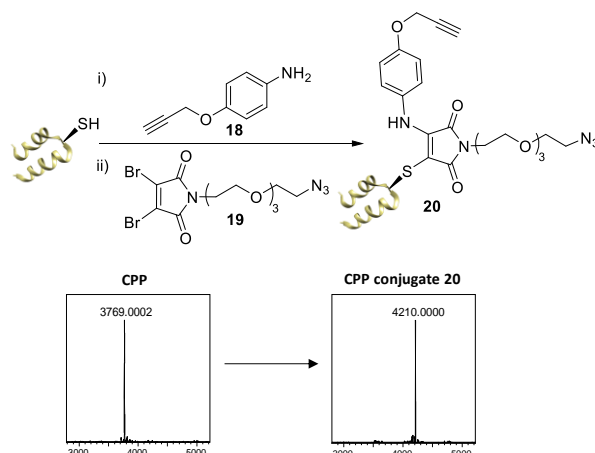


Figure 5: Dual thiol-amine conjugation on CPP. CPP (0.9 mM) in PB (10 mM, pH 6.5), amine-alkyne **18** (40 equiv.), DBM **19** (1.2 equiv.) left at 37 °C for 30 min; see **ESI Fig. S19-S22** for more detail.

The creation of clickable triconjugates was also carried out on two different proteins. DBM **21**, functionalised with biotin as a model handle (used widely in affinity purification and biological assays) was conjugated to mHSA, before amine-azide **22** (20 equiv.) was added and left at room temperature. The dual-functionalization occurred in 2 h at room temperature, with a small amount of unmodified and doubly modified HSA present as the only other detectable species (**Fig. 6**). Green Fluorescent Protein (GFP) was chosen as a second single cysteine containing protein to further appraise this dual conjugation strategy. Gratifyingly, conjugation of DBM **21** to the thiol was complete in just 5 minutes and amine-azide **22** in a further 95 minutes, with LCMS analysis showing a high degree of conversion (**Fig. 6**). Modification of conjugates **23** and **25** with DBCO-TAMRA through SPAAC successfully yielded the desired fluorescent-biotinylated dual conjugates **24** and **26** (**ESI Fig. S15** and **S18**). These procedures serve as examples to show the simplicity of forming useful triconjugates with thiol-amine dual conjugation. Similar bifunctional biotin-TAMRA conjugates have recently been used to observe protein modification in human cells.^{39,40}

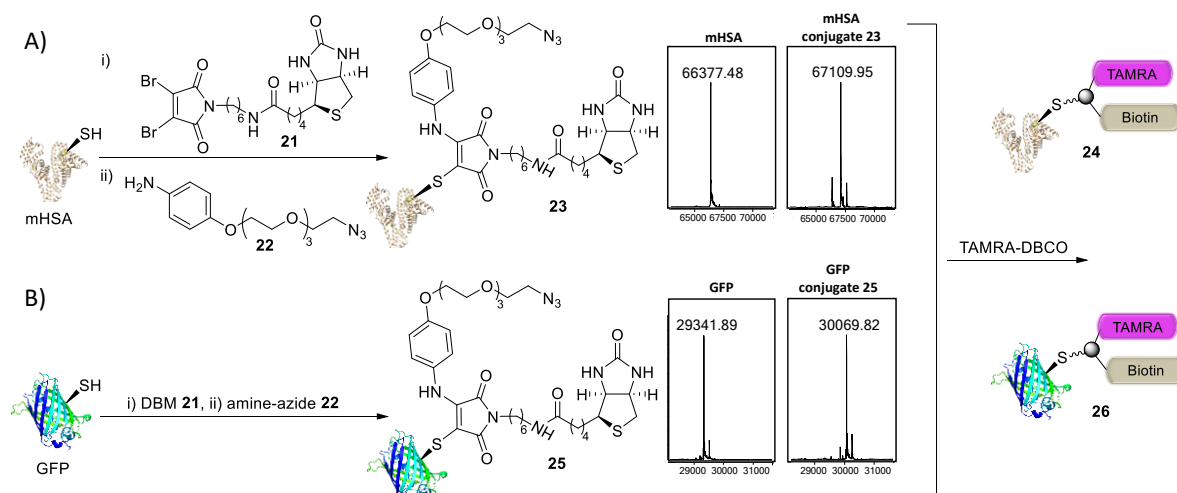


Figure 6: Trifunctional conjugate formation with mHSA and GFP (LCMS analysis shown). (A) (i) DBM **21** (1.5 equiv.), mHSA (100 μ M) in PBS pH 7.4 at RT for 45 min; (ii) amine-azide **22** (20 equiv.), RT for 2 h; (iii) TAMRA-DBCO (10 equiv.), RT for 16 h. (B) (i) DBM **21** (2.0 equiv.), GFP (40 μ M) in PBS pH 7.4 at 25 $^{\circ}$ C for 5 min; (ii) amine-azide **22** (30 equiv.), 25 $^{\circ}$ C for 95 min; (iii) TAMRA-DBCO (24 equiv.), RT for 5 h. See ESI Fig. S14-S18 for more detail.

Conclusion

We have demonstrated a dual thiol-amine bioconjugation strategy that provides distinct advantages over the hydrolysis of maleimide conjugates. This method simultaneously confers thiol-stability and enables addition of an extra functional handle. Unlike maleimide conjugation and hydrolysis, this method does not form regio- or stereoisomeric products. The dual conjugation reactions may be followed in real time using UV-vis spectroscopy owing to the formation of the aminothiomaleimide scaffold (ϵ_{415} 4250 M^{-1}). More specifically, we have demonstrated the use of *p*-anisidine-based functional amines that react with the bromothiomaleimide backbone and created trifunctional conjugates on both proteins and peptides. This thiol-amine coupling is also demonstrated to be a rare example of an efficient multi-component bioconjugation reaction on a protein.

Author Information

Corresponding Authors

j.r.baker@ucl.ac.uk, v.chudasama@ucl.ac.uk

Acknowledgements

The authors gratefully acknowledge EPSRC and Albumedix for funding and the EPSRC UK National Mass Spectrometry Facility (NMSF), Swansea. In the

Department of Chemistry, UCL, we also thank Kersti Karu and Malgorzata Puchnarewicz for assistance with mass spectrometry analysis, Rachael Dickman for HPLC assistance, and Abil Aliev for NMR analysis.

References

- 1 J. M. J. M. Ravasco, H. Faustino, A. Trindade and P. M. P. Gois, *Chem. - A Eur. J.*, 2019, **25**, 43–59.
- 2 K. Renault, J. W. Fredy, P. Y. Renard and C. Sabot, *Bioconjug. Chem.*, 2018, **29**, 2497–2513.
- 3 P. Adumeau, S. K. Sharma, C. Brent and B. M. Zeglis, *Mol. Imaging Biol.*, 2016, **18**, 1–17.
- 4 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, London, 3rd edn., 2013.
- 5 A. Beck, L. Goetsch, C. Dumontet and N. Corvaia, *Nat. Rev. Drug Discov.*, 2017, **16**, 315–337.
- 6 J. R. Junutula, H. Raab, S. Clark, S. Bhakta, D. D. Leipold, S. Weir, Y. Chen, M. Simpson, S. P. Tsai, M. S. Dennis, Y. Lu, Y. G. Meng, C. Ng, J. Yang, C. C. Lee, E. Duenas, J. Gorrell, V. Katta, A. Kim, K. McDorman, K. Flagella, R. Venook, S. Ross, S. D. Spencer, W. Lee Wong, H. B. Lowman, R. Vandlen, M. X. Sliwkowski, R. H. Scheller, P. Polakis and W. Mallet, *Nat. Biotechnol.*, 2008, **26**, 925–932.
- 7 E. Dolci, V. Froidevaux, C. Joly-duhamel, R. Auvergne, B. Boutevin and S. Caillol, *Polym. Rev.*, 2016, **56**, 512–556.
- 8 Z. Yang, Y. Guo, S. L. Ai, S. X. Wang, J. Z. Zhang, Y. X. Zhang, Q. C. Zou and H. X. Wang, *Mater. Chem. Front.*, 2019, **3**, 571–578.
- 9 B.-Q. Shen, K. Xu, L. Liu, H. Raab, S. Bhakta, M. Kenrick, K. L. Parsons-Reponte, J. Tien, S.-F. Yu, E. Mai, D. Li, J. Tibbitts, J. Baudys, O. M. Saad, S. J. Scales, P. J. McDonald, P. E. Hass, C. Eigenbrot, T. Nguyen, W. A. Solis, R. N. Fuji, K. M. Flagella, D. Patel, S. D. Spencer, L. A. Khawli, A. Ebens, W. L. Wong, R. Vandlen, S. Kaur, M. X. Sliwkowski, R. H. Scheller, P. Polakis and J. R. Junutula, *Nat. Biotechnol.*, 2012, **30**, 184–189.
- 10 A. D. Baldwin and K. L. Kiick, *Bioconjug. Chem.*, 2011, **22**, 1946–1953.
- 11 P. A. Szijj, C. Bahou and V. Chudasama, *Drug Discov. Today Technol.*, 2018, **30**, 27–34.
- 12 S. D. Fontaine, R. Reid, L. Robinson, G. W. Ashley and D. V. Santi, *Bioconjug. Chem.*, 2015, **26**, 145–152.
- 13 L. N. Tumey, M. Charati, T. He, E. Sousa, D. Ma, X. Han, T. Clark, J. Casavant, F. Loganzo, F. Barletta, J. Lucas and E. I. Graziani, *Bioconjug. Chem.*, 2014, **25**, 1871–1880.
- 14 M. E. B. Smith, M. B. Caspersen, E. Robinson, M. Morais, A. Maruani, J. P. M. Nunes, K. Nicholls, M. J. Saxton, S. Caddick, J. R. Baker and V. Chudasama, *Org. Biomol. Chem.*, 2015, **13**, 7946–7949.
- 15 M. Morais, J. P. M. Nunes, K. Karu, N. Forte, I. Benni, M. E. B. Smith, S. Caddick, V. Chudasama and J. R. Baker, *Org. Biomol. Chem.*, 2017, **15**, 2947–2952.
- 16 M. W. Jones, R. A. Strickland, F. F. Schumacher, S. Caddick, J. R. Baker, M. I. Gibson and D. M. Haddleton, *J. Am. Chem. Soc.*, 2012, **134**, 1847–1852.
- 17 Y. Cui, Y. Yan, Y. Chen and Z. Wang, *Macromol. Chem. Phys.*, 2013, **214**,

- 470–477.
- 18 J. K. Y. Tan, J. L. Choi, H. Wei, J. G. Schellinger and S. H. Pun, *Biomater. Sci.*, 2015, **3**, 112–120.
 - 19 N. Forte, M. Livanos, E. Miranda, M. Morais, X. Yang, V. S. Rajkumar, K. A. Chester, V. Chudasama and J. R. Baker, *Bioconjug. Chem.*, 2018, **29**, 486–492.
 - 20 M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick and J. R. Baker, *J. Am. Chem. Soc.*, 2010, **132**, 1960–1965.
 - 21 A. Wall, K. Nicholls, M. B. Caspersen, S. Skrivergaard, K. A. Howard, K. Karu, V. Chudasama and J. R. Baker, *Org. Biomol. Chem.*, 2019, **17**, 7870–7873.
 - 22 A. B. Mabire, M. P. Robin, W. Quan, H. Willcock, V. G. Stavros and R. K. O. Reilly, *Chem. Commun.*, 2015, **51**, 9733–9736.
 - 23 Y. Xie, J. T. Husband, M. Torrent-Sucarrat, H. Yang, W. Liu and R. K. O'Reilly, *Chem. Commun.*, 2018, **54**, 3339–3342.
 - 24 F. Brotzel, C. C. Ying and H. Mayr, *J. Org. Chem.*, 2007, **72**, 3679–3688.
 - 25 A. Dirksen, T. M. Hackeng and P. E. Dawson, *Angew. Chem. Int. Ed.*, 2006, **45**, 7581–7584.
 - 26 B. Elsadek and F. Kratz, *J. Control. Release*, 2012, **157**, 4–28.
 - 27 R. E. Kontermann, *Expert Opin. Biol. Ther.*, 2016, **16**, 903–915.
 - 28 D. Sleep, J. Cameron and L. R. Evans, *Biochim. Biophys. Acta - Gen. Subj.*, 2013, **1830**, 5526–5534.
 - 29 M. T. Larsen, M. Kuhlmann, M. L. Hvam and K. A. Howard, *Mol. Cell. Ther.*, 2016, **4**, 1–12.
 - 30 K. K. Schelde, K. Nicholls, F. Dagnæs-Hansen, K. Bunting, H. Rawsthorne, B. Andersen, C. J. A. Finnis, M. Williamson, J. Cameron and K. A. Howard, *J. Biol. Chem.*, 2019, **294**, 3735–3743.
 - 31 M. Fernández, A. Shamsabadi and V. Chudasama, *Chem. Commun.*, 2020, **56**, 1125–1128.
 - 32 S. Wang, H. Ma, J. Li, X. Chen, Z. Bao and S. Sun, *Talanta*, 2006, **70**, 518–521.
 - 33 L. Ducry and B. Stump, *Bioconjug. Chem.*, 2010, **21**, 5–13.
 - 34 S. Santra, C. Kaitanis, O. J. Santiesteban and J. M. Perez, *J. Am. Chem. Soc.*, 2011, **133**, 16680–16688.
 - 35 K. Kurrikoff, M. Gestin and Ü. Langel, *Expert Opin. Drug Deliv.*, 2016, **13**, 373–387.
 - 36 F. Wang, Y. Wang, X. Zhang, W. Zhang, S. Guo and F. Jin, *J. Control. Release*, 2014, **174**, 126–136.
 - 37 C. Bechara and S. Sagan, *FEBS Lett.*, 2013, **587**, 1693–1702.
 - 38 J. E. Dixon, G. Osman, G. E. Morris, H. Markides, M. Rotherham, Z. Bayoussef, A. J. El Haj, C. Denning and K. M. Shakesheff, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, E291–E299.
 - 39 M. Broncel, R. A. Serwa, P. Ciepla, E. Krause, M. J. Dallman, A. I. Magee and E. W. Tate, *Angew. Chem. Int. Ed.*, 2015, **54**, 5948–5951.
 - 40 E. M. Storck, J. Morales-Sanfrutos, R. A. Serwa, N. Panyain, T. Lanyon-Hogg, T. Tolmachova, L. N. Ventimiglia, J. Martin-Serrano, M. C. Seabra, B. Wojciak-Stothard and E. W. Tate, *Nat. Chem.*, 2019, **11**, 552–561.

Amine-Thiol Dual Conjugation Manuscript.pdf (1.38 MiB)

[view on ChemRxiv](#) • [download file](#)

Electronic Supplementary Information

**One-Pot Thiol-Amine Bioconjugation to Maleimides;
Simultaneous Stabilisation and Dual Functionalisation**

*Archie Wall,[†] Alfie G. Wills,[†] Nafsika Forte,[†] Calise Bahou,[†] Lisa Bonin,[†] Karl
Nicholls,[‡] Michelle T. Ma,[§] Vijay Chudasama^{†,¶,*} and James R. Baker^{†,*}*

[†] Department of Chemistry, University College London, 20 Gordon Street, London,
WC1H 0AJ, UK

[‡] Alumedix Ltd, Mabel Street, Nottingham, NG2 3ED, UK

[§] School of Biomedical Engineering and Imaging Sciences, King's College London,
St Thomas' Hospital, London SE1 7EH, UK

[¶] Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy,
Universidade de Lisboa, Lisbon, Portugal

GENERAL REMARKS	3
SYNTHESIS OF COMPOUNDS.....	5
AMINE REACTIONS IN ORGANIC SOLVENT.....	12
AMINE REACTIONS IN BUFFER.....	29
SYNTHESIS OF FUNCTIONALISED DBMS	34
SYNTHESIS OF <i>P</i>-ANISIDINE ANALOGUES.....	38
NMR REACTIONS.....	51
PROTEIN AND PEPTIDE MASS SPECTRA	53
Native WT HSA	55
Native mHSA	56
Expression and Reduction of GFP	57
Native CPP	58
WT HSA, <i>N</i> -Me DBM (14)	59
mHSA, <i>N</i> -Me DBM (15)	60
WT HSA, <i>N</i> -Me DBM, <i>p</i> -Anisidine (16).....	61
mHSA, <i>N</i> -Me DBM, <i>p</i> -Anisidine (17)	63
mHSA, <i>N</i> -Me DBM, <i>p</i> -Anisidine (<i>in situ</i>) (17)	65
mHSA, Biotin DBM, Amine-Azide (23)	66
mHSA, Biotin DBM, Amine-Azide, TAMRA-DBCO (24)	67
GFP, Biotin DBM, Amine-Azide, TAMRA-DBCO (25 and 26)	68
CPP, Azide DBM, Amine-Alkyne (<i>in situ</i>) (20).....	71
STABILITY EXPERIMENTS.....	75
Small Molecule Study	75
Thiol Stability of Conjugate 17	79
References	86

General Remarks

Materials:

All commercially available chemicals were acquired from either Sigma-Aldrich or Alfa-Aesar and used without further purification. Recombinant Human Serum Albumin (WT HSA) was provided by Alumedix (Recombunin® Elite, batch no. AX190201). Albumin mutant C34A + K93C (mHSA) was kindly provided by Alumedix, having been expressed according to literature.¹ All buffer solutions were prepared with doubly deionised water, degassed with argon and filtered before use. Phosphate-buffered saline (PBS) was 137 mM NaCl and 12 mM sodium phosphates at pH 7.4. DBM-azide **19** was synthesised according to literature.²

Methods:

All organic syntheses were carried out at atmospheric pressure, under argon. All bioconjugation reactions were carried out at atmospheric pressure in 1.5 ml Eppendorf tubes. Room temperature is defined as 20-22 °C.

Centrifugation was carried out with an Eppendorf 5415 R centrifuge, at 12.6 RPM.

Reactions were analysed by thin layer chromatography (TLC), with Merck KGaA silica gel 60 F₂₅₄ TLC plates. TLC plates were visualised with a UVLS-26 EL series UV lamp, at 254 or 365 nm. Ninhydrin, Dragendorff's reagent, potassium permanganate and iodine were used as stains.

Flash chromatography was carried out on a Biotage Isolera One 3.0, using Graceresolv or Biotage normal phase columns. Where dry loading was used, the sample was adsorbed onto Geduran SI 60 silica gel (40-63 µm).

¹H and ¹³C NMR analysis was carried out on a Bruker Avance Neo 700 equipped with a 5 mm helium-cooled broadband cryoprobe or a Bruker Avance III 600 spectrometer equipped with a DCH cryoprobe. ¹H experiments were carried out at 700 or 600 MHz and ¹³C experiments at 151 MHz. Unless otherwise stated all NMR experiments were carried out at room temperature. All NMR analysis was carried out in the deuterated solvent system stated.

Infra-red spectra were recorded on a Bruker Platinum ALPHA FT-IR spectrometer operating in attenuated total reflection (ATR) mode. Absorptions are characterized as s (sharp), br (broad), m (medium), w (weak).

Melting points were recorded for all solids at room temperature with a Gallenkamp digital Melting-point apparatus 5A 6797. All melting points are uncorrected.

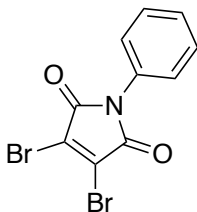
High and low resolution (HRMS/LRMS) mass spectra acquired with electrospray ionisation (ES/ES+/ES-) modes were obtained at UCL with Agilent 6510 QTOF. High and low resolution (HRMS/LRMS) mass spectra acquired with nanospray (NSI) ionisation modes were obtained at The National Mass Spectrometry Facility Swansea on a Thermo Scientific LTQ Orbitrap XL.

Protein LCMS was performed with an LC system connected to an Agilent 6510 Q-TOF spectrometer. A PLRP-s, 1000 Å, 8 µM column was used. Peptide LCMS was performed on a Waters Acquity UPLC connected to Waters Acquity Single Quad Detector (SQD). A Hypersil Gold C4, 1.9 µm, 2.1 µm × 50 µm column was used.

UV analysis was carried out with either a Cary UV spectrophotometer and recorded with Cary WinUV software, or a Shimadzu UV-2600 spectrophotometer with UVprobe software. A quartz cuvette with a 1 cm path length was used to hold samples. Unless stated, all UV scan experiments were carried out at room temperature. All samples were baseline corrected. UV Kinetics experiments were carried out with Cary Kinetics software and unless stated all kinetics experiments were carried out at 25 °C: absorbance data at 375 and 415 nm were obtained, with data timepoints at 415 nm obtained at 2.6 seconds after the respective timepoint at 375 nm. NB: This small time difference was not taken into account in the kinetics graph for GFP (). Data points acquired when amine was being added to the cuvette (outside the machine) have been omitted. For clarity, the 375 nm data was plotted until a plateau was observed, upon which point the 415 nm data was plotted to show the progress of amine addition. The 415 nm plot was then stopped when a plateau was observed. Where experiments have been left in the UV machine for longer (due to running overnight experiments, for example) before MS analysis, this has been indicated in the text.

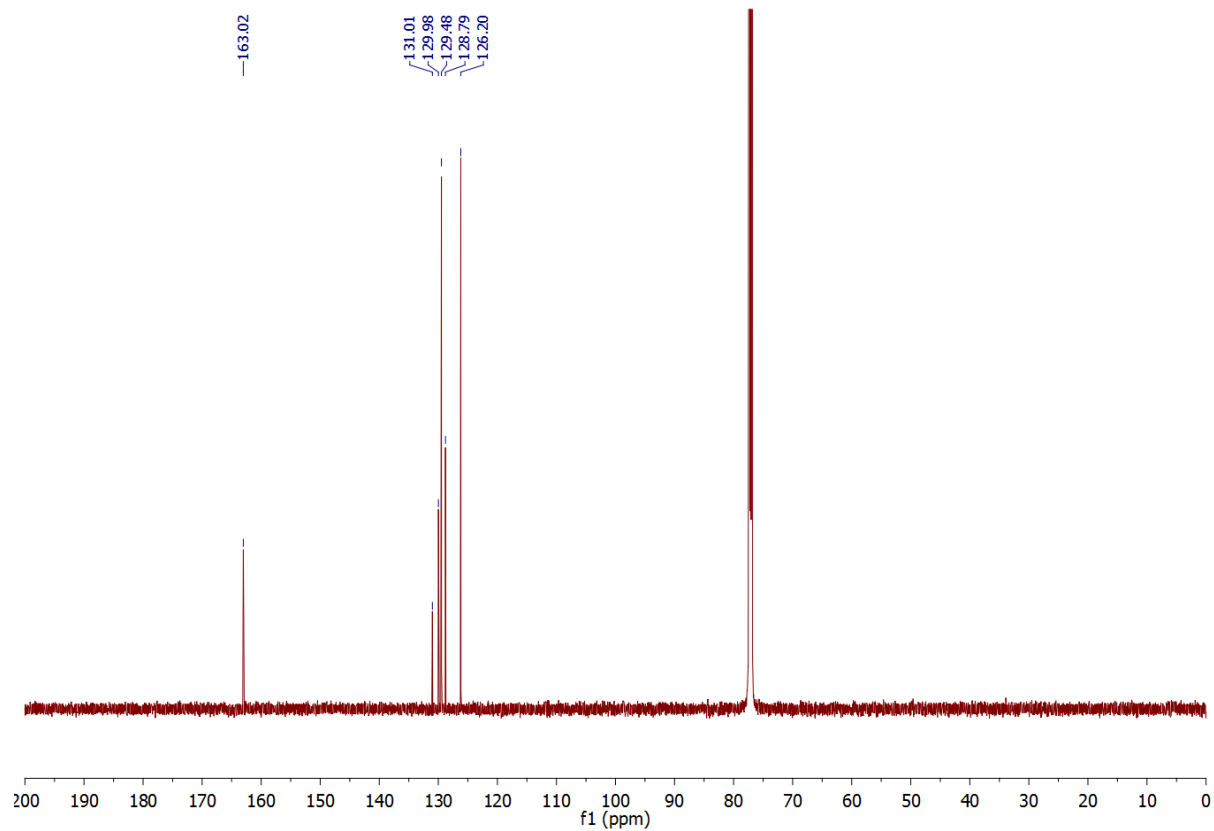
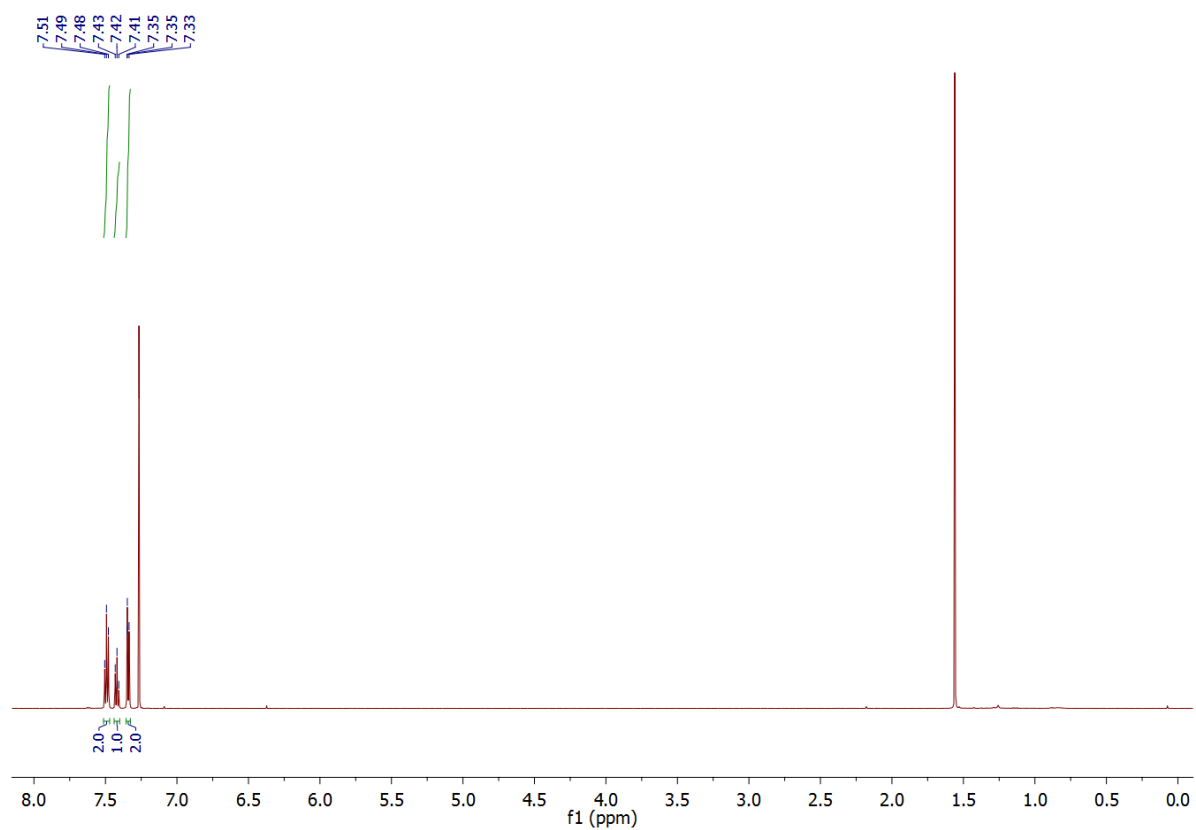
Synthesis of Compounds

Synthesis of 3,4-dibromo-1-phenyl-1*H*-pyrrole-2,5-dione³ (**27**)

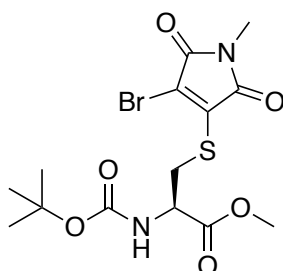


To *N*-phenylmaleimide (0.430 g, 2.50 mmol) and sodium acetate (0.615 g, 7.50 mmol) dissolved in acetic acid (25 mL), bromine (0.28 mL, 5.50 mmol) was added dropwise and the resultant solution heated under reflux for 30 minutes (120 °C). The solvent was removed *in vacuo* and the remaining solid dissolved in EtOAc (90 mL) and washed with 20% aqueous Na₂S₂O₃ (40 mL × 3), saturated NaHCO₃ (40 mL × 2) and brine (50 mL). The organic phase was dried over MgSO₄ and the solvent removed *in vacuo* to yield the crude product. Purification by column chromatography (1-20% EtOAc/petroleum ether) yielded the product as a faint yellow powder (0.722 g, 2.18 mmol, 87%).

m.p. 161.0-164.0 °C (lit. 162-163 °C)³; ¹H NMR (600 MHz, CDCl₃); δ 7.49 (t, *J* = 7.8 Hz, 2H, CH), 7.42 (t, *J* = 7.5 Hz, 1H, CH), 7.34 (d, *J* = 7.2 Hz, 2H, CH); ¹³C NMR (151 MHz, CDCl₃); δ 163.0 (CO), 131.1 (C), 130.0 (C), 129.5 (CH), 128.8 (CH), 126.2 (CH); IR (solid); 1740 (s), 2950 (w) cm⁻¹.

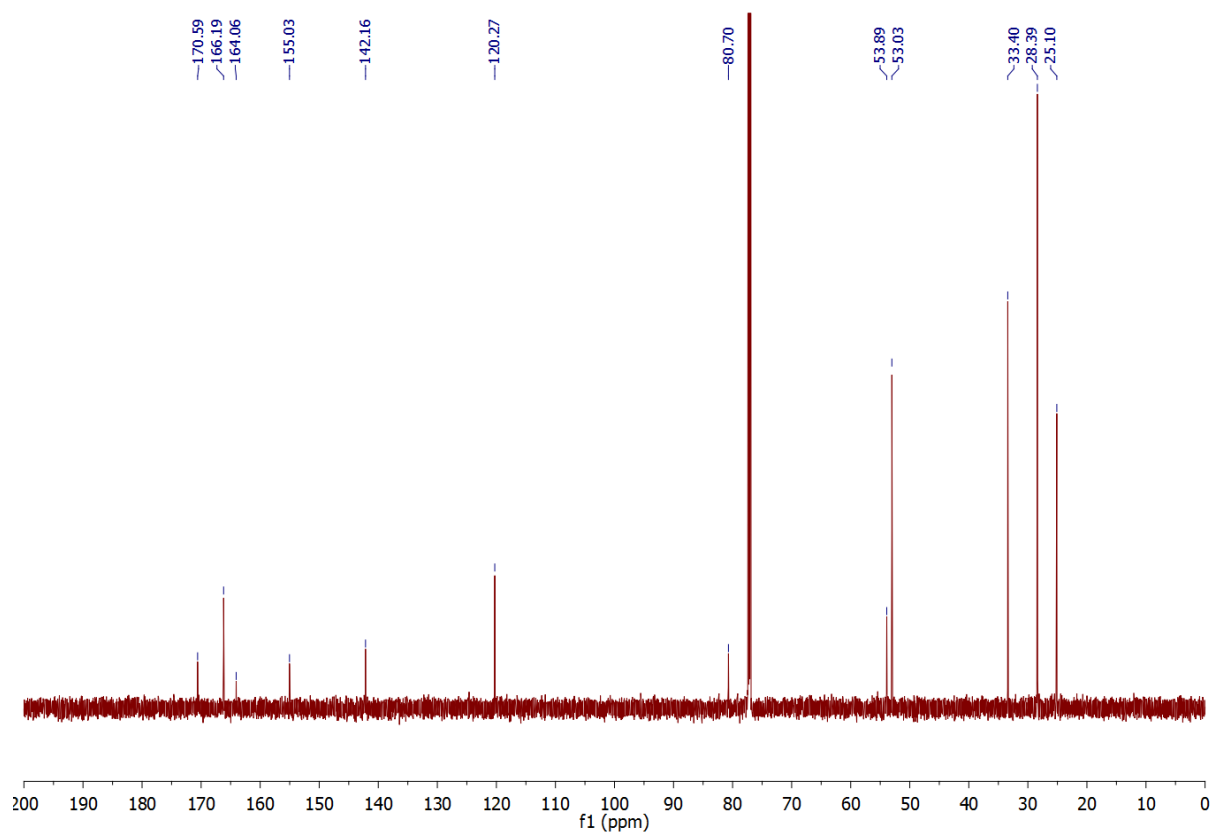
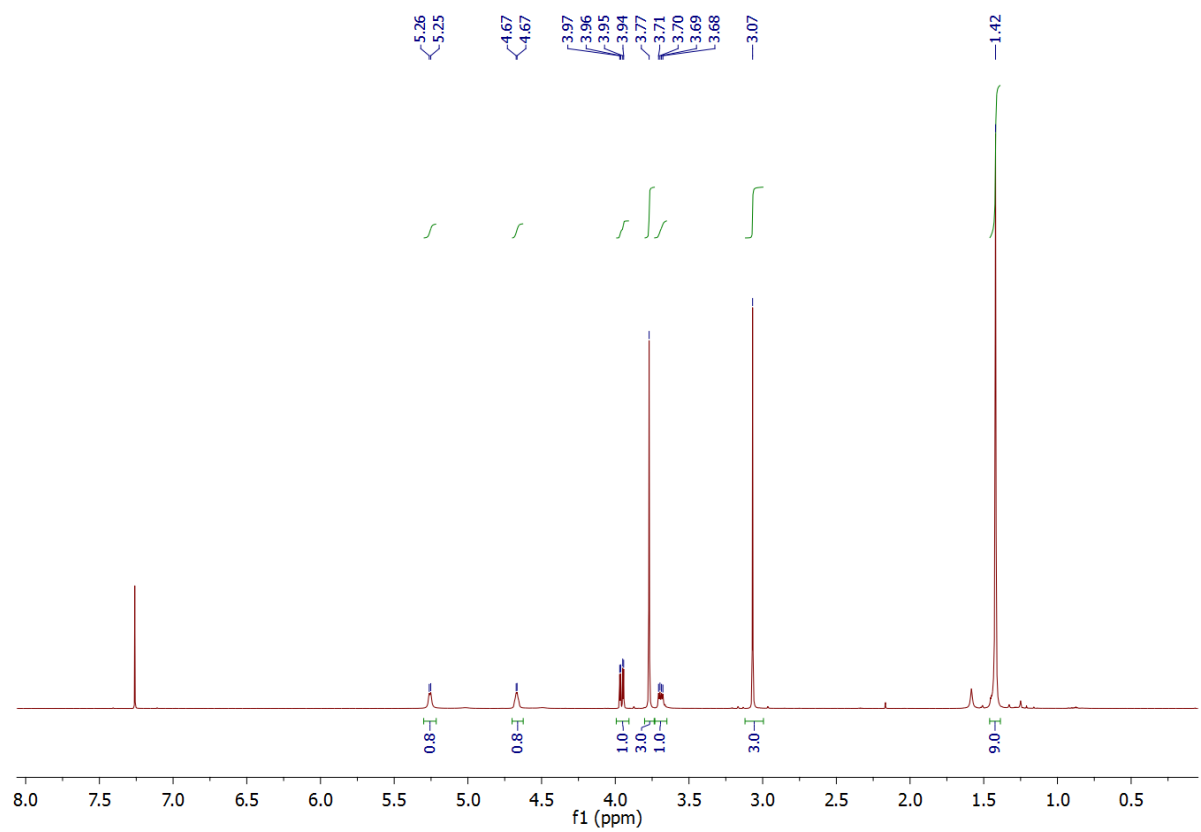


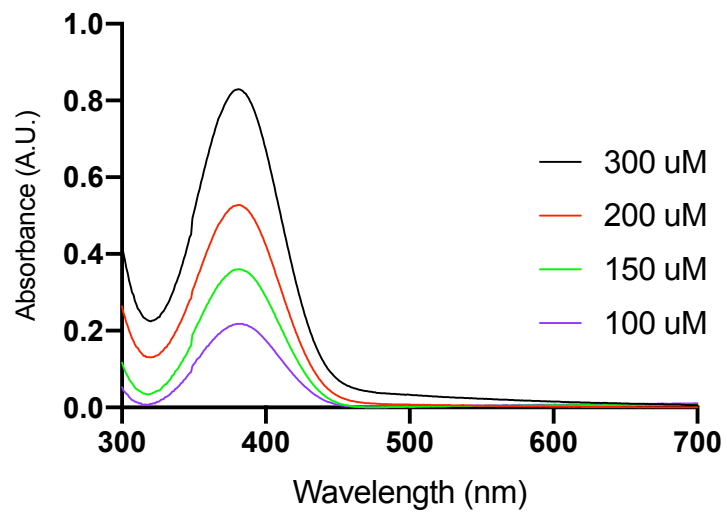
Synthesis of methyl S-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-N-(tert-butoxycarbonyl)-L-cysteinate (**6**)



To 3,4-dibromo-1-methyl-1H-pyrrole-2,5-dione (0.343 g, 1.27 mmol) and sodium acetate (0.055 g, 0.669 mmol) dissolved in methanol (100 mL), methyl (*tert*-butoxycarbonyl)-L-cysteinate (0.150 g, 0.637 mmol) dissolved in methanol (20 mL) was added dropwise over 90 minutes and the resultant solution stirred at room temperature for 30 minutes. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-100% EtOAc/petroleum ether) yielded the product as a yellow oil that crystallised upon standing (0.222 g, 0.524 mmol, 82%).

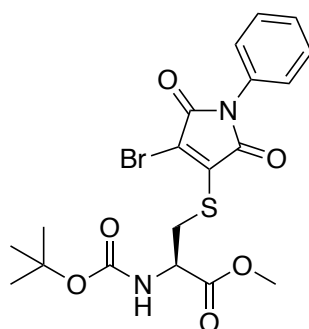
m.p. 126.0-129.0 °C; ^1H NMR (600 MHz, CDCl_3); δ 5.26 (d, J = 7.2 Hz, 1H, CONH), 4.67 (d, J = 4.4 Hz, 1H, HNCHCO), 3.95 (dd, J = 14.1, 4.5 Hz, 1H, SCHH), 3.77 (s, 3H, OCH_3), 3.69 (dd, J = 14.0, 6.7 Hz, 1H, SCHH), 3.07 (s, 3H, NCH_3), 1.42 (s, 9H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (151 MHz, CDCl_3); δ 170.6 (CO), 166.2 (CO), 164.1 (CO), 155.0 (CO), 142.2 (CS), 120.3 (BrC), 80.7 (C), 53.9 (CH), 53.0 (CH_3), 33.4 (CH_2), 28.4 (CH_3), 25.1 (CH_3); IR (solid); 1740 (s), 2950 (w), 3350 (m) cm^{-1} ; LRMS (ES^+) 323.0 (60, $[\text{M}^{79}\text{Br}+\text{H}-\text{Boc}]^+$), 325.0 (60, $[\text{M}^{81}\text{Br}+\text{H}-\text{Boc}]^+$) 423.0 (65, $[\text{M}^{79}\text{Br}+\text{H}]^+$), 425.0 (60, $[\text{M}^{81}\text{Br}+\text{H}]^+$); HRMS (EI) calcd for $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_6\text{SBr}$ $[\text{M}^{79}\text{Br}+\text{H}]^+$ 423.0226, observed 423.0222.





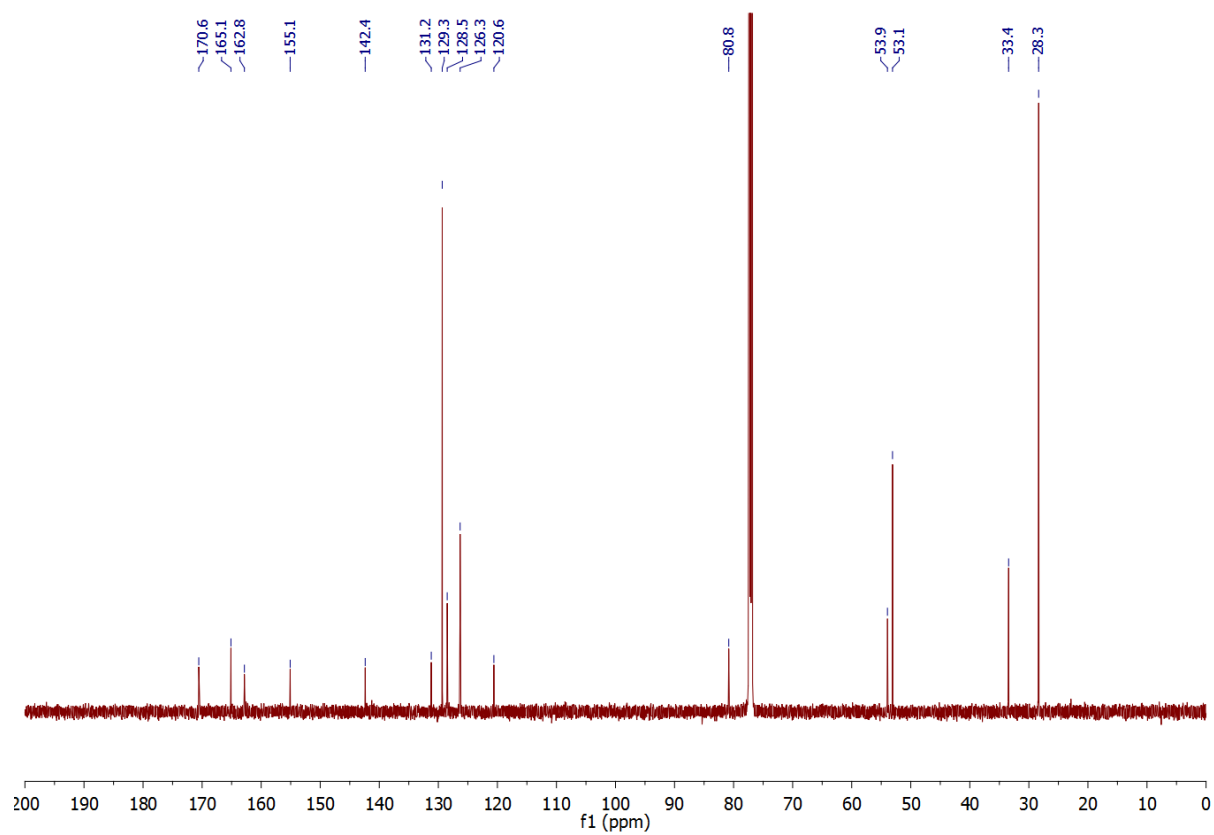
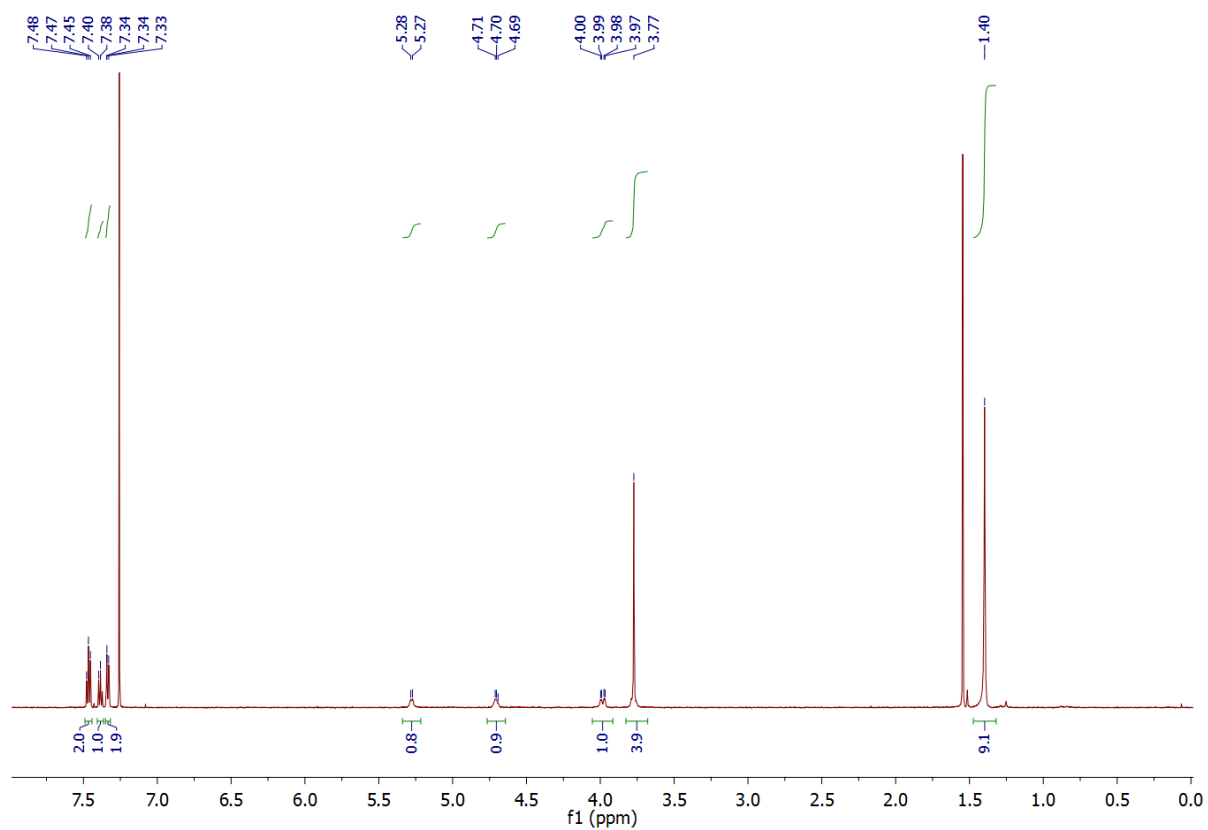
Absorbance spectra recorded in 10% THF in PBS (pH 7.4).

Synthesis of S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1H-pyrrol-3-yl)-N-(*tert*-butoxycarbonyl)-L-cysteinate (**5**)



To 3,4-dibromo-1-phenyl-1H-pyrrole-2,5-dione (0.294 g, 0.888 mmol) and sodium acetate (0.048 g, 0.583 mmol) dissolved in methanol (75 mL), methyl (*tert*-butoxycarbonyl)-L-cysteinate (0.131 g, 0.555 mmol) dissolved in methanol (20 mL) was added dropwise over 90 minutes. The resultant solution was stirred for a further 30 minutes. A colour change from pale yellow to yellow was observed. The solvent was removed *in vacuo* and the crude product purified by column chromatography (0-100% EtOAc/petroleum ether) to yield the product as a yellow powder (0.220 g, 0.453 mmol, 82%);

m.p. 139.0-141.0 °C; ^1H NMR (600 MHz, CDCl_3); δ 7.47 (t, J = 9.0 Hz, 2H, ArH), 7.39 (t, J = 9.0 Hz, 1H, ArH), 7.34 (d, J = 6.0 Hz, 2H, ArH), 5.28 (d, J = 7.8 Hz, 1H, NH), 4.70 (dd, J = 11.7, 7.2 Hz, 1H, HNC HCO), 3.98 (dd, J = 14.1 Hz, 4.2 Hz, 1H, SCH HCH), 3.77 (br s, 4H, OCH_3 /SCH HCH), 1.40 (s, 9H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (151 MHz, CDCl_3); δ 170.6 (CO), 165.1 (CO), 162.8 (CO), 155.1 (CO), 142.4 (CS), 131.2 (ArC), 129.3 (ArC), 128.5 (ArC), 126.3 (ArC), 120.6 (BrC), 80.8 (C), 53.9 (CH), 53.1 (CH_3), 33.4 (CH_2), 28.3 (CH_3); IR (solid); 1735 (s), 2900 (w), 3400 (m) cm^{-1} ; LRMS (ES^+) 485.0 (100, $[\text{M}^{79}\text{Br}+\text{H}]^+$), 487.0 (100, $[\text{M}^{81}\text{Br}+\text{H}]^+$); HRMS (EI) calcd for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}_6\text{SBr}$ $[\text{M}^{79}\text{Br}+\text{H}]^+$ 485.0376, observed 485.0363.



Amine Reactions in Organic Solvent

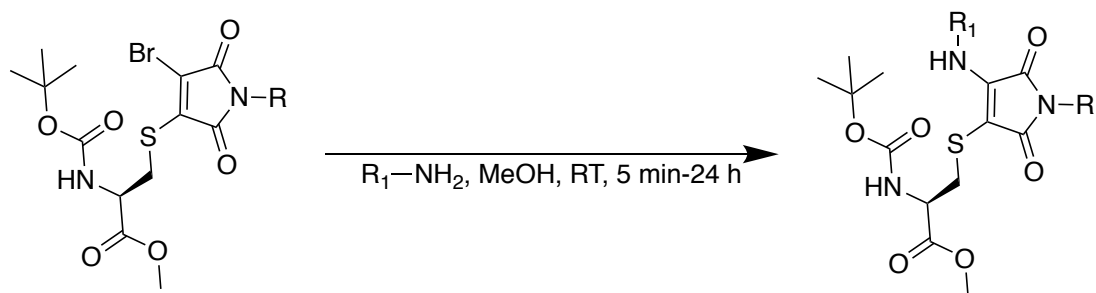
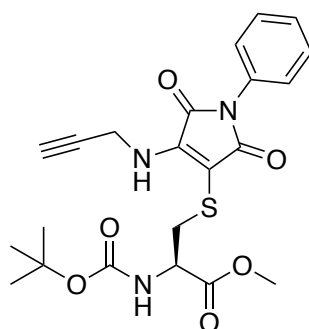


Table S1: Amine reactions in methanol.

R	R ¹ -(Amine)	Equiv. of amine	Time [†]	Yield
Ph	Propargylamine	2	24 h*	53%
Ph	Piperidine	2	20 min	98%
Ph	Piperidine	5	10 min	99%
Ph	Pyrrolidine	2	15 min	96%
Ph	Pyrrolidine	5	5 min	99%
Me	Pyrrolidine	2	10 min	92%
Me	Aniline	2	24 h	82%
Me	<i>p</i> -anisidine	2	24 h	90%
Me	<i>N</i> -Me <i>p</i> -anisidine	2	24 h	88%

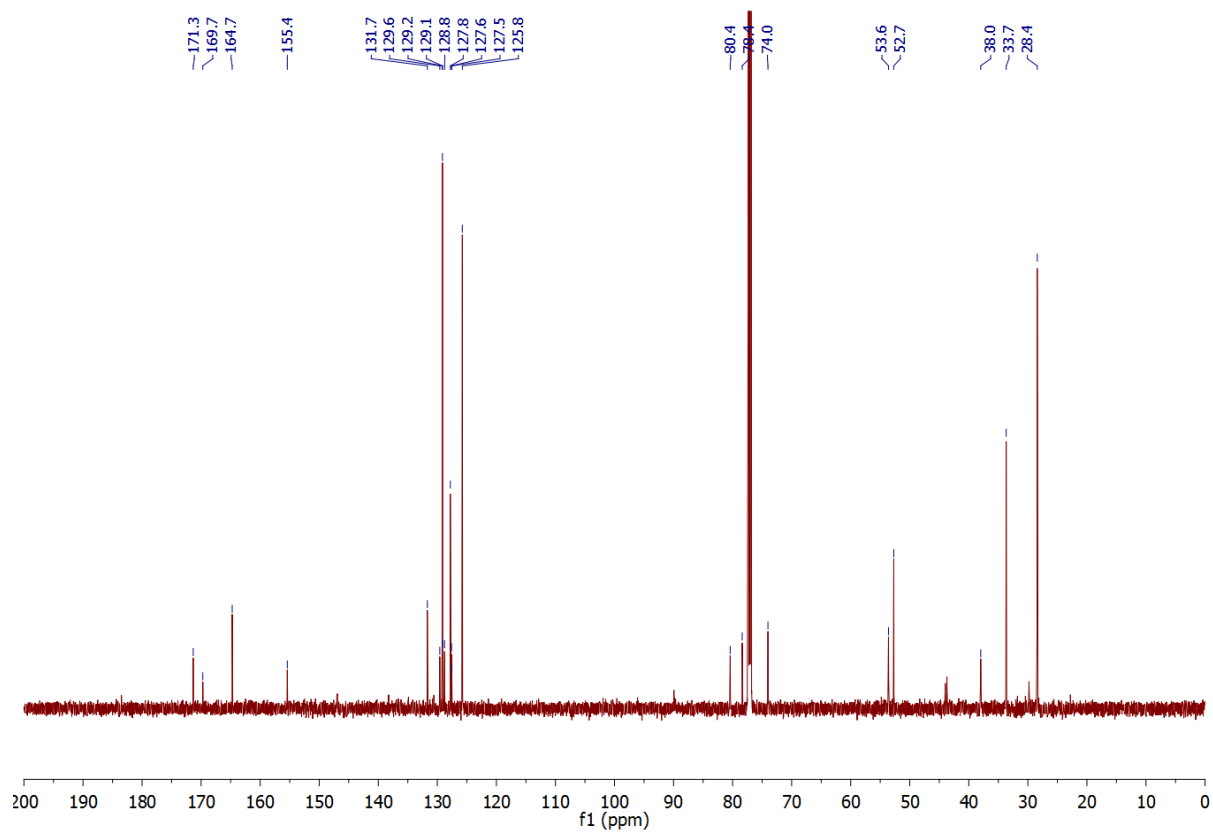
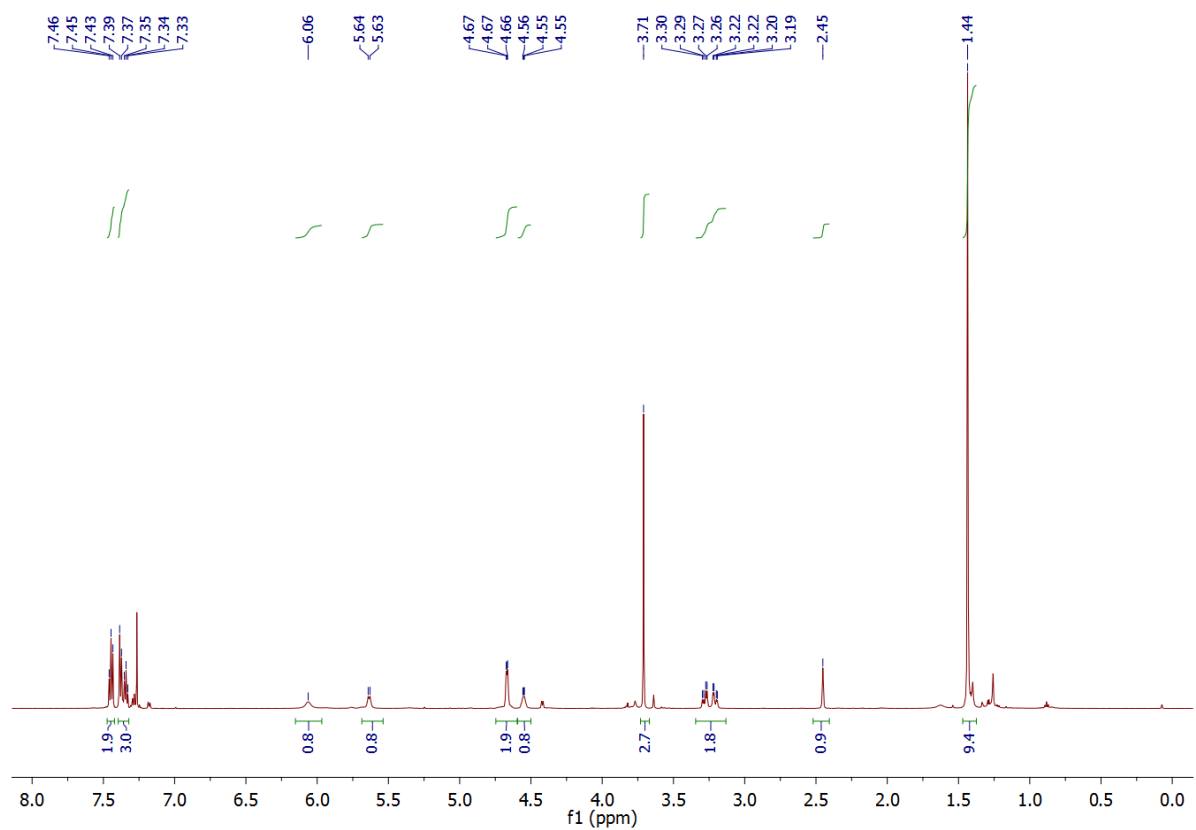
All R=Me reactions are 21 mg **6** in 24 mL (2.1 mM) final volume, R=Ph reactions are all different conc. (2.1-7.8 mM of **5**). † Reaction times measured by TLC. * TLC analysis not carried out before 24 h.

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(2,5-dioxo-1-phenyl-4-(prop-2-yn-1-ylamino)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**28**)

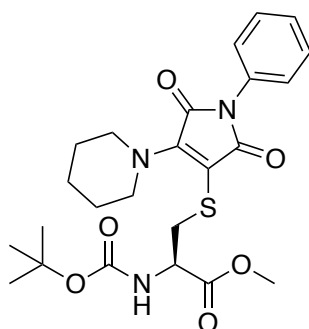


To *S*-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.036 g, 0.074 mmol) dissolved in methanol (10 mL), 3-amino-1-propyne (0.010 mL, 0.15 mmol) was added dropwise and the resultant solution stirred at room temperature for 24 hours. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as a yellow oil (0.018 g, 0.039 mmol, 53%).

^1H NMR (600 MHz, CDCl_3); δ 7.45 (t, $J = 7.8$ Hz, 2H, ArH), 7.38 (d, $J = 7.7$ Hz, 2H, ArH), 7.34 (t, $J = 7.3$ Hz, 1H, ArH), 6.06 (br s, 1H, NH), 5.63 (d, $J = 7.6$ Hz, 1H, NH), 4.67-4.66 (m, 2H, CH_2CCH), 4.56-4.55 (m, 1H, NHCHCO), 3.71 (s, 3H, OCH_3) 3.28 (dd, $J = 14.0, 5.7$ Hz, 1H, SCHHCH), 3.21 (dd, $J = 14.0, 4.2$ Hz, 1H, SCHHCH), 2.45 (s, 1H, CH_2CCH), 1.44 (s, 9H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (151 MHz, CDCl_3); δ 171.3 (CO), 169.7 (CO), 164.7 (CO), 155.4 (CO), 131.7 (C), 129.6 (C), 129.1 (C), 128.8 (C), 127.6 (C), 125.8 (C), 80.4 (C), 79.4 (C), 74.0 (C), 53.6 (C), 52.7 (CH_3), 38.0 (CH_2), 33.7 (CH_2), 28.4 (CH_3); IR (thin film); 1600 (s), 2960 (w), 3300 (m) cm^{-1} ; LRMS (ES^+) 482.1 (100, $[\text{M}+\text{Na}]^+$), 460.2 (95, $[\text{M}+\text{H}]^+$); HRMS (ES^+) calcd for $\text{C}_{22}\text{H}_{26}\text{N}_3\text{O}_6\text{S}$ $[\text{M}+\text{H}]^+$ 460.1542, observed 460.1540.

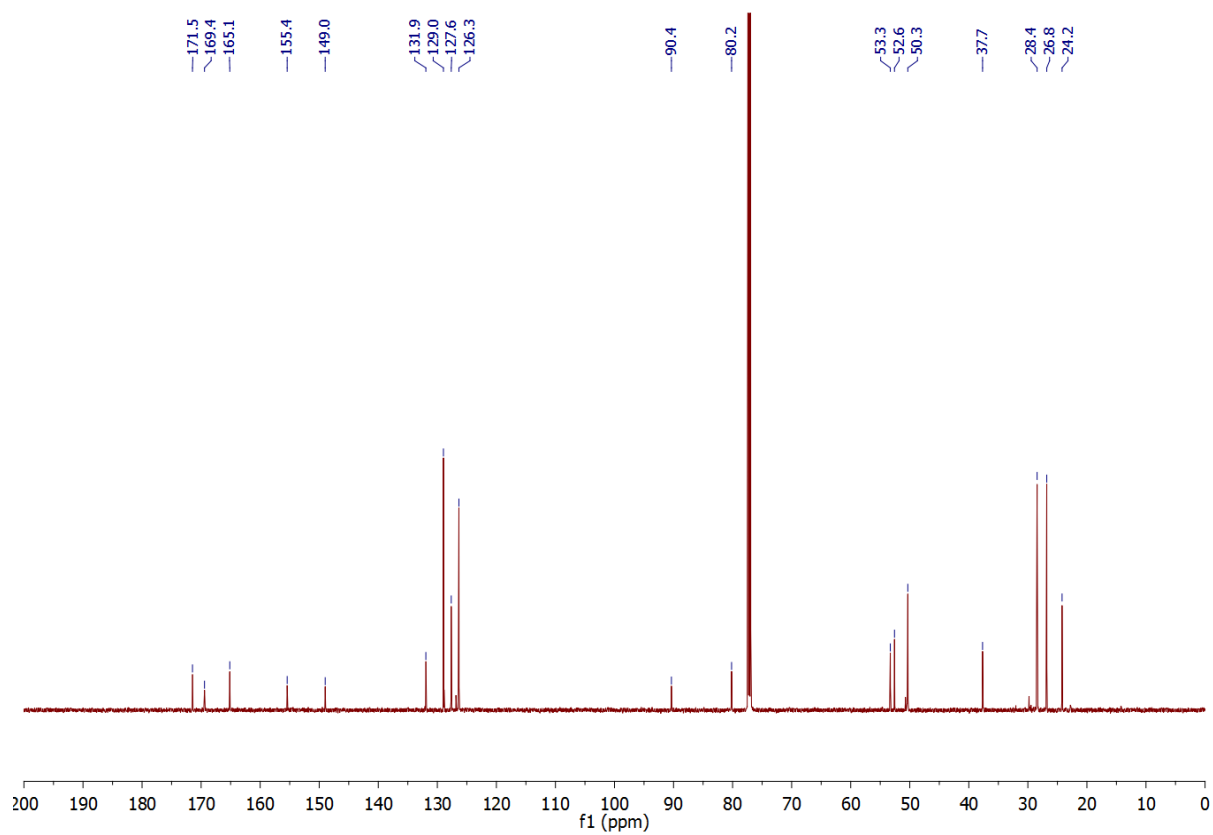
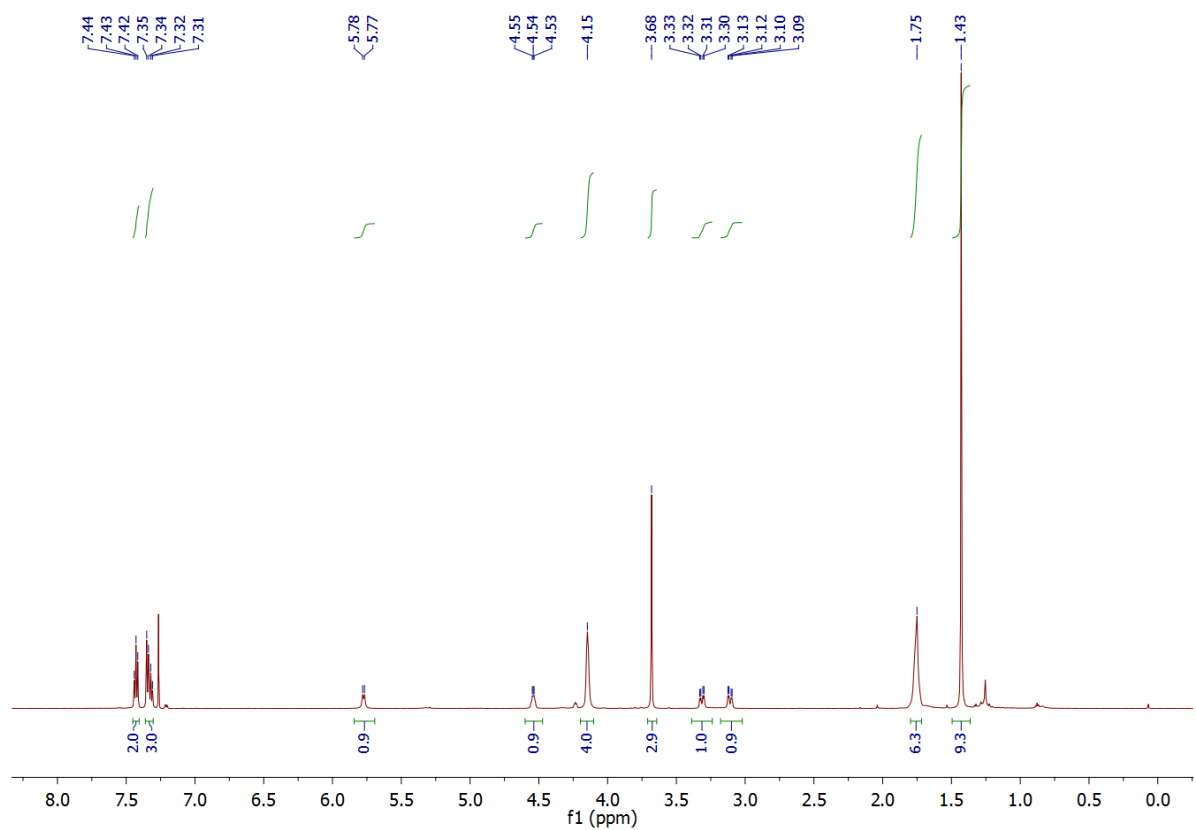


Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-phenyl-4-(piperidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-L-cysteinate (**7**)

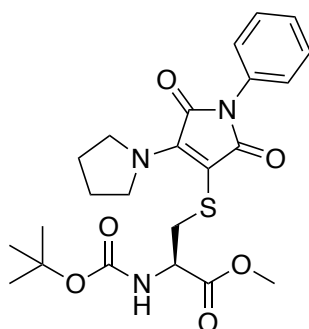


To S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-L-cysteinate (0.024 g, 0.050 mmol) dissolved in methanol (15 mL), piperidine (0.001 mL, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred at room temperature for 20 minutes. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.024 g, 0.049 mmol, 98%). *N.B.* Same protocol followed for 5 equiv. experiment shown in table S1.

¹H NMR (600 MHz, CDCl₃); δ 7.43 (t, *J* = 7.8 Hz, 2H, ArH), 7.35-7.31 (m, 3H, ArH), 5.77 (d, *J* = 7.9 Hz, 1H, NH), 4.54 (dt, *J* = 8.4, 4.8 Hz, 1H, NHCHCO), 4.15 (br s, 4H, N(CH₂)₂), 3.68 (s, 3H, OCH₃), 3.31 (dd, *J* = 13.9, 4.9 Hz, 1H, SCHHCH), 3.11 (dd, *J* = 13.9, 4.4 Hz, 1H, SCHHCH), 1.76-1.74 (m, 6H, N(CH₂)₂(CH₂)₃), 1.43 (s, 9H, C(CH₃)₃); ¹³C NMR (151 MHz, CDCl₃); δ 171.5 (CO), 169.4 (CO), 165.1 (CO), 155.4 (CO), 149.0 (CN), 131.9 (ArC), 129.0 (ArC), 127.6 (ArC), 126.3 (ArC), 90.4 (CS), 80.2 (C), 53.3 (CH₃), 52.6 (CH), 50.3 (CH₂), 37.7 (CH₂), 28.4 (CH₃), 26.8 (CH₂), 24.2 (CH); IR (thin film); 1650 (s), 2980 (m), 3250 (w) cm⁻¹; LRMS (ES⁺) 490.1 (70, [M+H]⁺); HRMS (ES⁺) calcd for C₂₄H₃₂N₃O₆S [M+H]⁺ 490.1934, observed 490.1479.

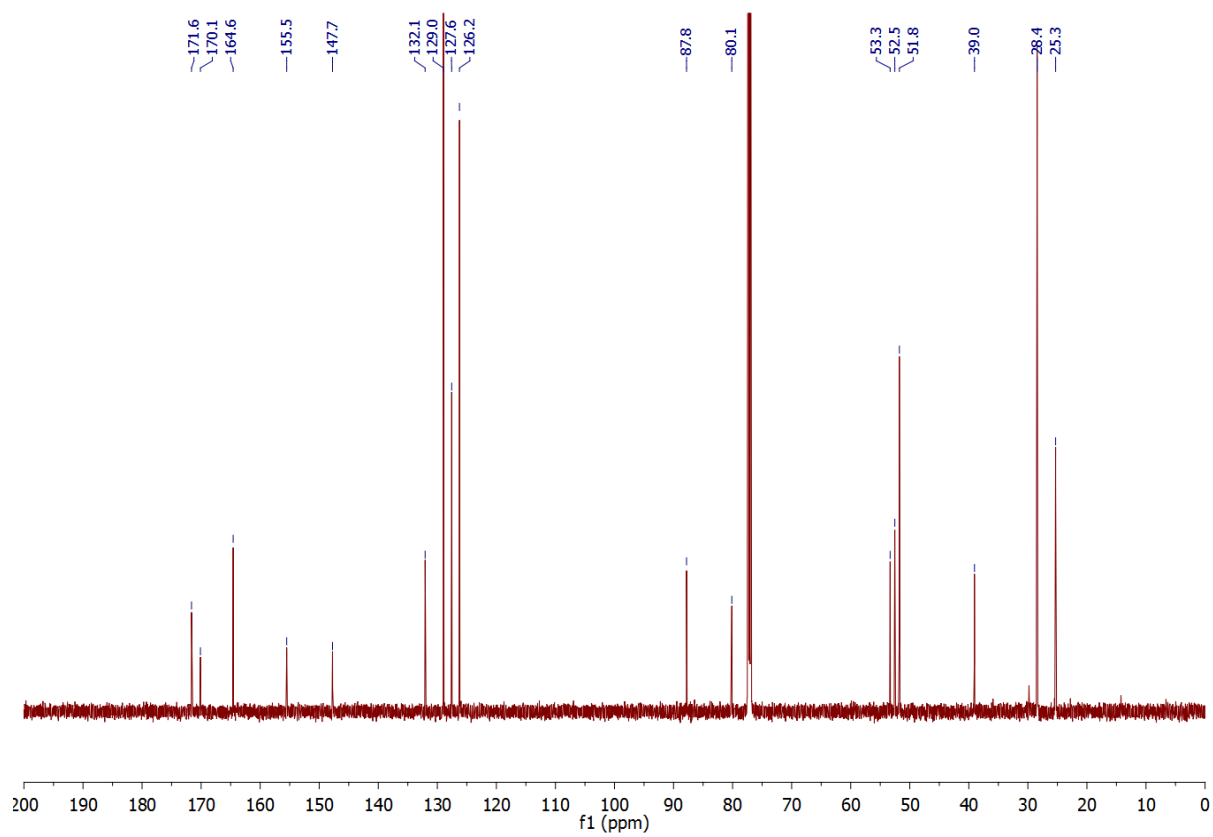
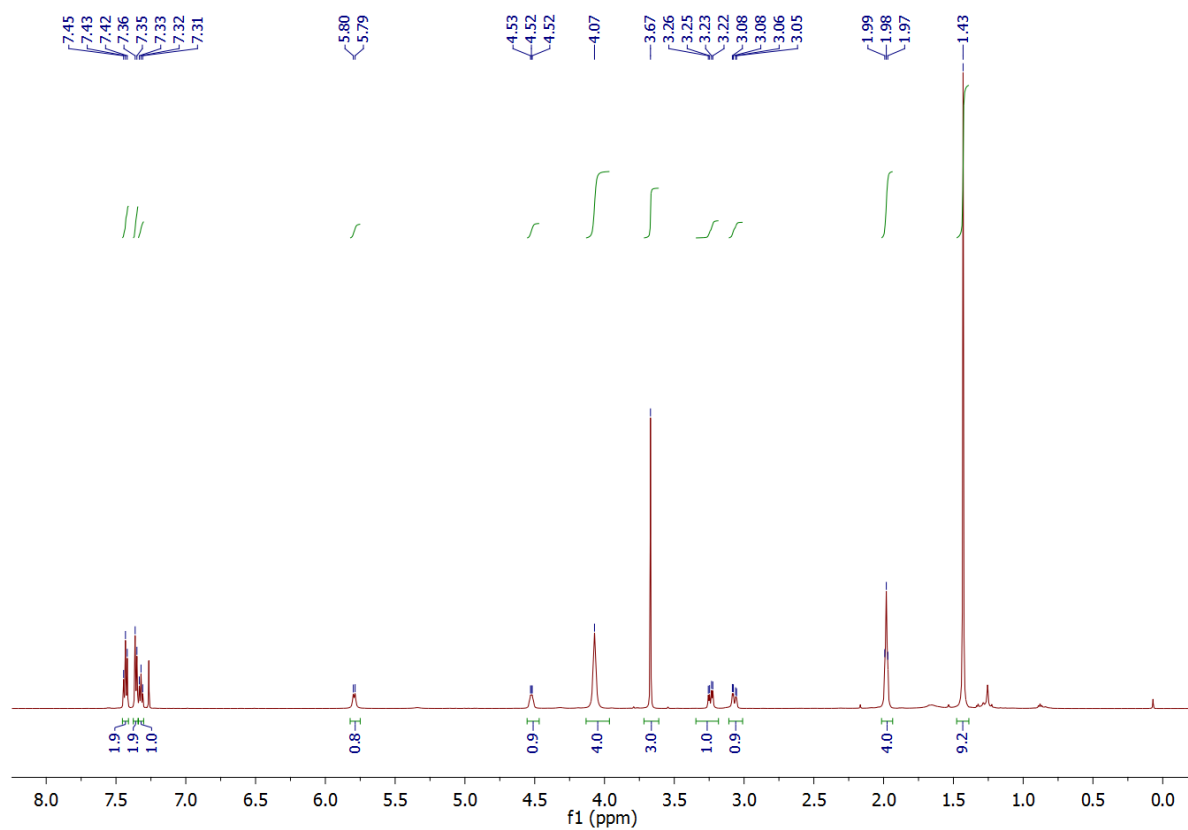


Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-phenyl-4-(pyrrolidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**8**)

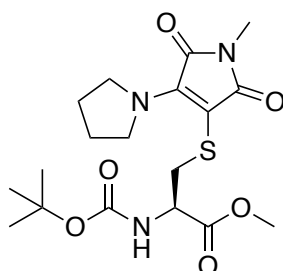


To S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.024 g, 0.050 mmol) dissolved in methanol (20 mL), pyrrolidine (0.007 g, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 15 minutes at room temperature. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil that crystallised upon standing (0.023 g, 0.048 mmol, 96%). *N.B. Same protocol followed for 5 equiv. experiment shown in table S1.*

m.p. 127.0-131.0 °C; ¹H NMR (600 MHz, CDCl₃); δ 7.43 (t, *J* = 7.8 Hz, 2H, ArH), 7.36 (d, *J* = 7.6 Hz, 2H, ArH), 7.32 (t, *J* = 7.4 Hz, 1H, ArH), 5.79 (d, *J* = 7.9 Hz, 1H, NH), 4.52 (dt, *J* = 8.4, 4.6 Hz, 1H, NHCHCO), 4.07 (br s, 4H, N(CH₂)₂), 3.67 (s, 3H, OCH₃), 3.24 (dd, *J* = 13.9, 5.5 Hz, 1H, SCHHCH), 3.07 (dd, *J* = 13.9, 4.2 Hz, 1H, SCHHCH), 1.98 (t, *J* = 6.5 Hz, 4H, N(CH₂)₂(CH₂)₂), 1.43 (s, 9H, C(CH₃)₃); ¹³C NMR (151 MHz, CDCl₃); δ 171.6 (CO), 170.1 (CO), 164.6 (CO), 155.5 (CO), 147.7 (CN), 132.1 (ArC), 129.0 (ArC), 127.6 (ArC), 126.2 (ArC), 87.8 (CS), 80.1 (C), 53.3 (CH₂), 52.5 (CH), 51.8 (CH₃), 39.0 (CH₂), 28.4 (CH₃), 25.3 (CH₂); IR (solid); 1550 (s), 2900 (w), 3440 (w) cm⁻¹; LRMS (ES⁺) 476.2 (100, [M+H]⁺); HRMS (ES⁺) calcd for C₂₃H₃₀N₃O₆S [M+H]⁺ 476.1855, observed 476.1854.

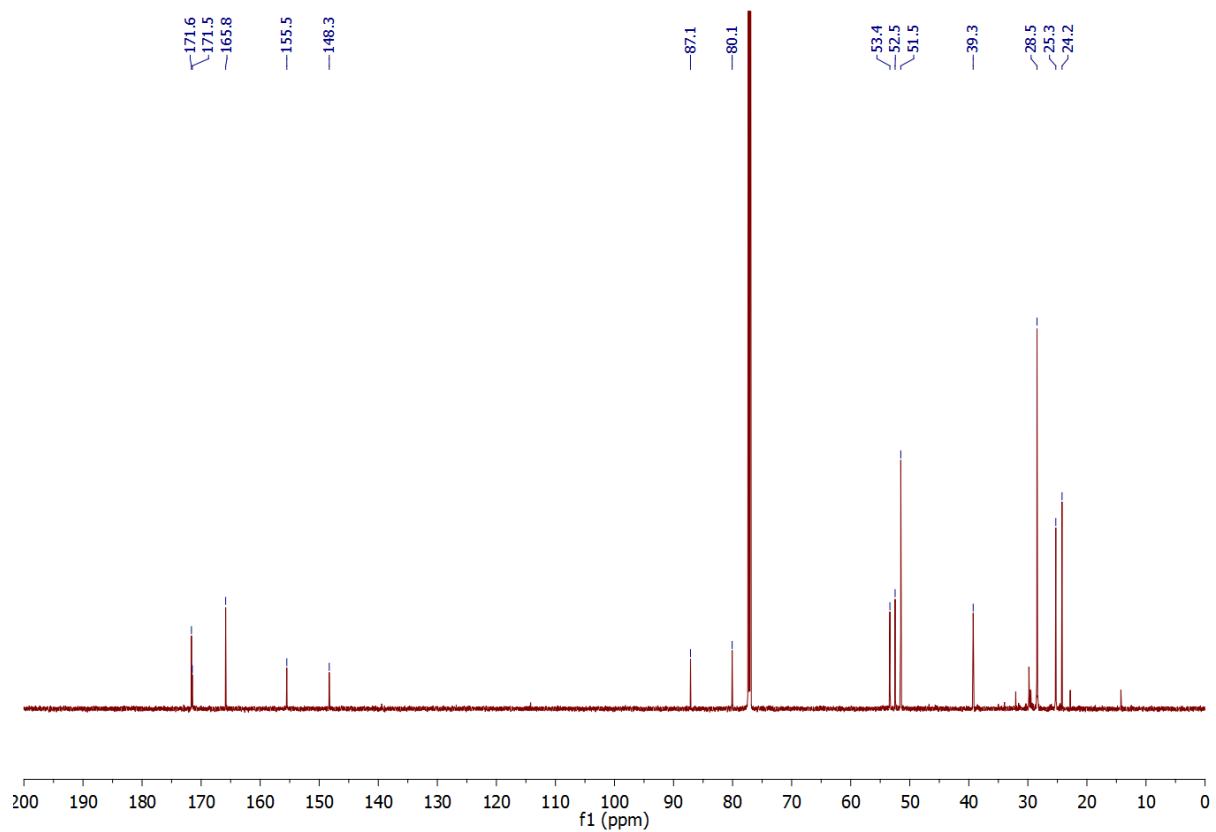
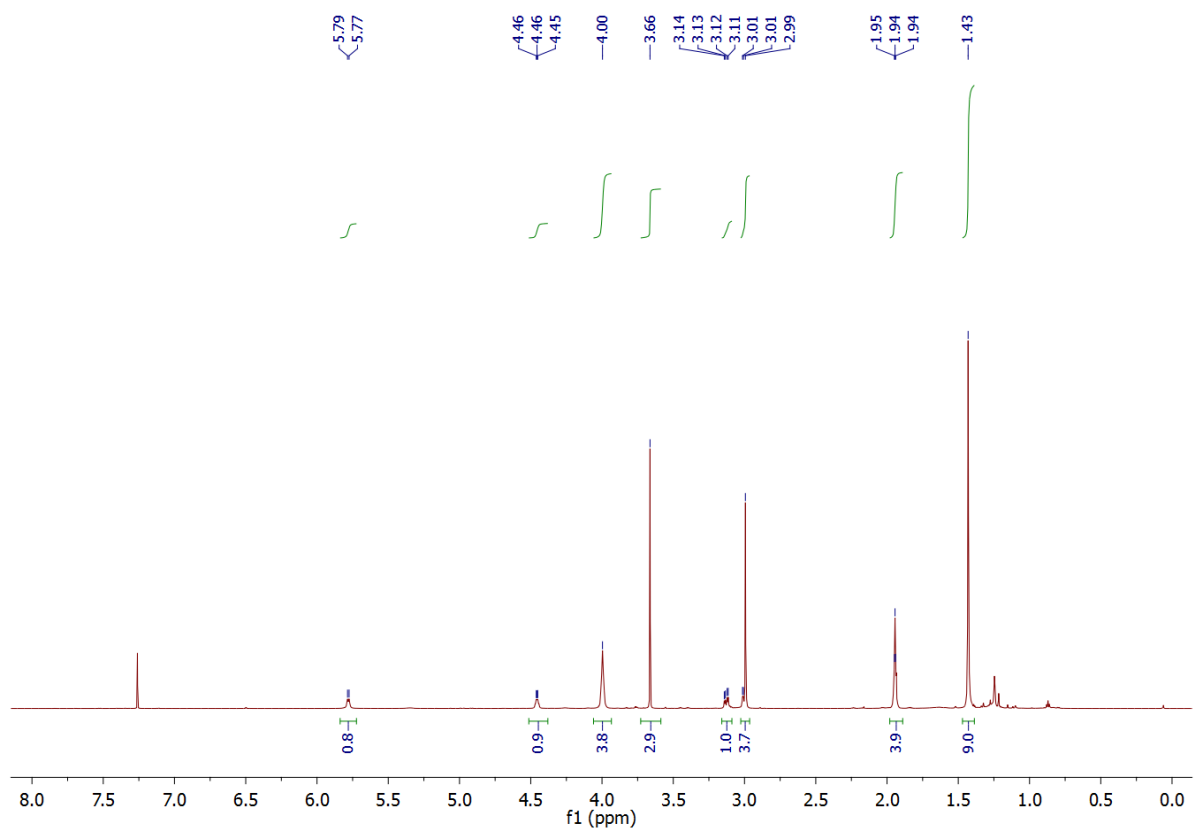


Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-methyl-4-(pyrrolidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**9**)

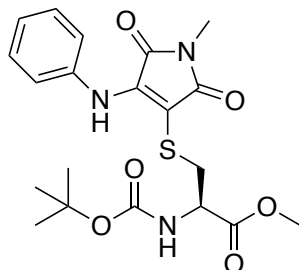


To methyl 3-((4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL) pyrrolidine (0.0083 mL, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise for 10 min and the resultant solution stirred for 10 min at room temperature. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/Cyclohexane) yielded the product as an orange oil (0.019 g, 0.046 mmol, 92%).

^1H NMR (600 MHz, CDCl_3); δ 5.78 (d, J = 7.6 Hz, 1H, NH), 4.46-4.45 (m, 1H, NHCHCO), 4.00 (br s, 4H, $\text{N}(\text{CH}_2)_2$), 3.66 (s, 3H, OCH_3), 3.13 (dd, J = 13.9, 5.5 Hz, 1H, SCHHCH), 3.01-2.99 (m, 4H, SCHH/NCH_3), 1.95-1.94 (m, 4H, $\text{N}(\text{CH}_2)_2(\text{CH}_2)_2$), 1.43 (s, 9H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (151 MHz, CDCl_3); δ 171.6 (CO), 171.5 (CO), 165.8 (CO), 155.5 (CO), 148.3 (CN), 87.1 (CS), 80.1 (C), 53.4 (CH_3), 52.5 (CH), 51.5 (CH_2), 39.3 (CH_2), 28.5 (CH_3), 25.3 (CH_2), 24.2 (CH_3); IR (thin film); 1600 (s), 2950 (w), 3350 (m) cm^{-1} ; LRMS (ES^+) 414.2 (100, $[\text{M}+\text{H}]^+$); HRMS (ES^+) calcd for $\text{C}_{18}\text{H}_{28}\text{N}_3\text{O}_6\text{S}$ $[\text{M}+\text{H}]^+$ 414.1699, observed 414.1705.

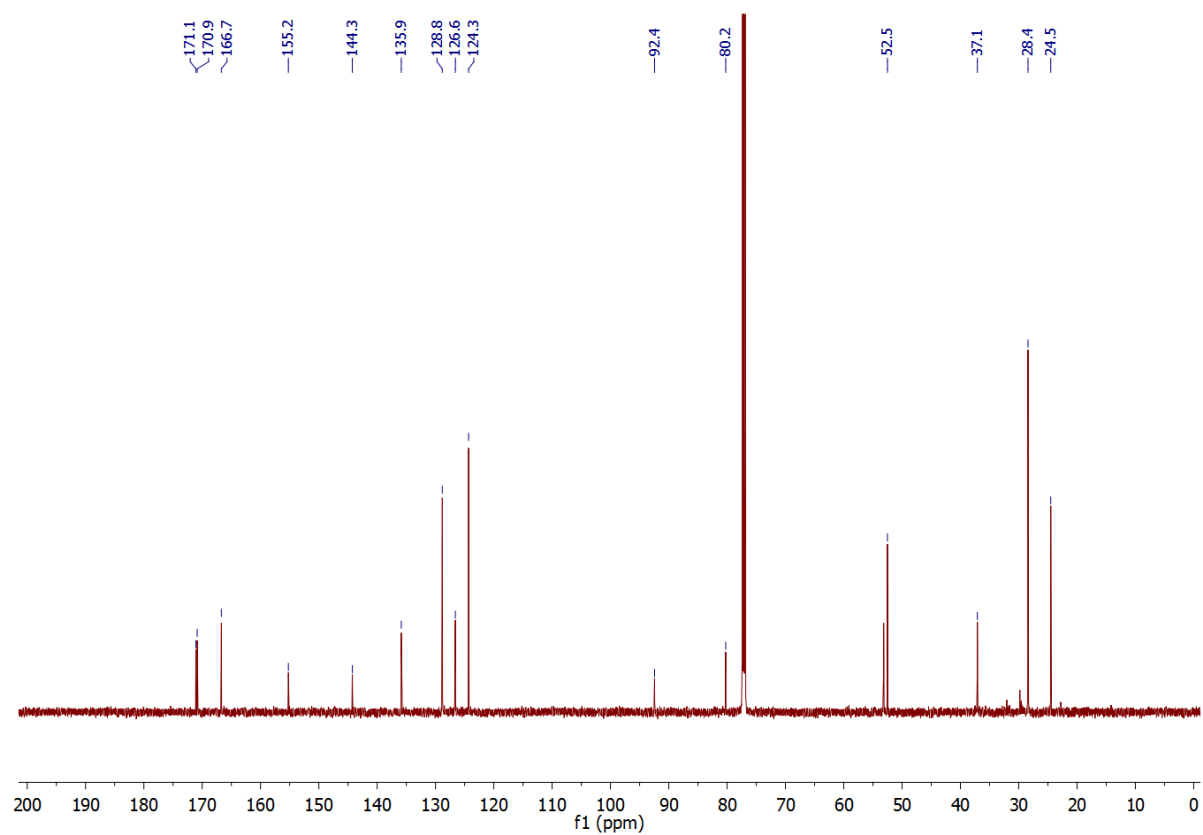
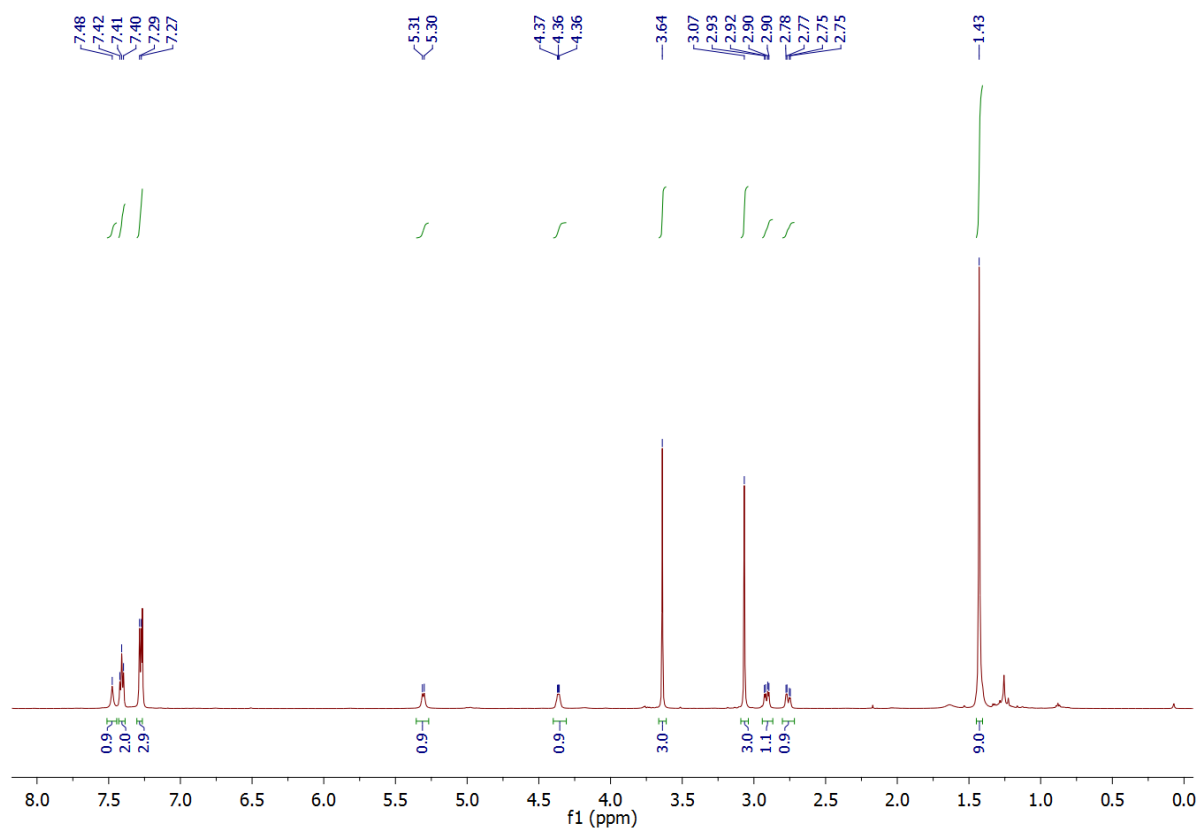


Synthesis of methyl *N*-(tert-butoxycarbonyl)-*S*-(1-methyl-2,5-dioxo-4-(phenylamino)-2,5-dihydro-1H-pyrrol-3-yl)-*L*-cysteinate (**10**)

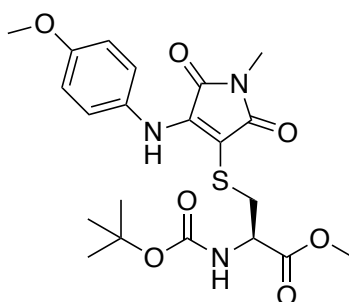


To methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-*N*-(tert-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL) aniline (0.009 mL, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 24 hours at room temperature. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.018 g, 0.041 mmol, 82%).

^1H NMR (600 MHz, CDCl_3); δ 7.48 (br s, 1H, ArNH), 7.41 (t, $J = 7.7$ Hz, 2H, ArH), 7.29-7.27 (m, 2H, ArH), 5.30 (d, $J = 7.8$ Hz, 1H, NH), 4.36 (dt, $J = 9.6, 4.8$ Hz, 1H, NHCHCO₂), 3.64 (s, 3H, OCH₃), 3.07 (s, 3H, NCH₃), 2.91 (dd, $J = 13.9, 5.1$ Hz, 1H SCHHCH), 2.76 (dd, $J = 13.9, 4.1$ Hz, 1H, SCHHCH), 1.43 (s, 9H, C(CH₃)₃); ^{13}C NMR (151 MHz, CDCl_3); δ 171.1 (CO), 170.9 (CO), 166.7 (CO), 155.2 (CO), 144.3 (C), 135.9 (C), 128.8 (C), 126.6 (C), 124.3 (C), 92.4 (CS), 80.2 (C), 53.2 (CH), 52.5 (CH₃), 37.1 (CH₂), 28.4 (CH₃), 24.5 (CH₃); IR (thin film); 1600 (s), 2950 (w), 3400 (w) cm^{-1} ; LRMS (ES^+) 458.2 (100, $[\text{M}+\text{Na}]^+$); HRMS (ES^+) calcd for $\text{C}_{20}\text{H}_{26}\text{N}_3\text{O}_6\text{S}$ $[\text{M}+\text{Na}]^+$ 458.1464, observed 458.1508.

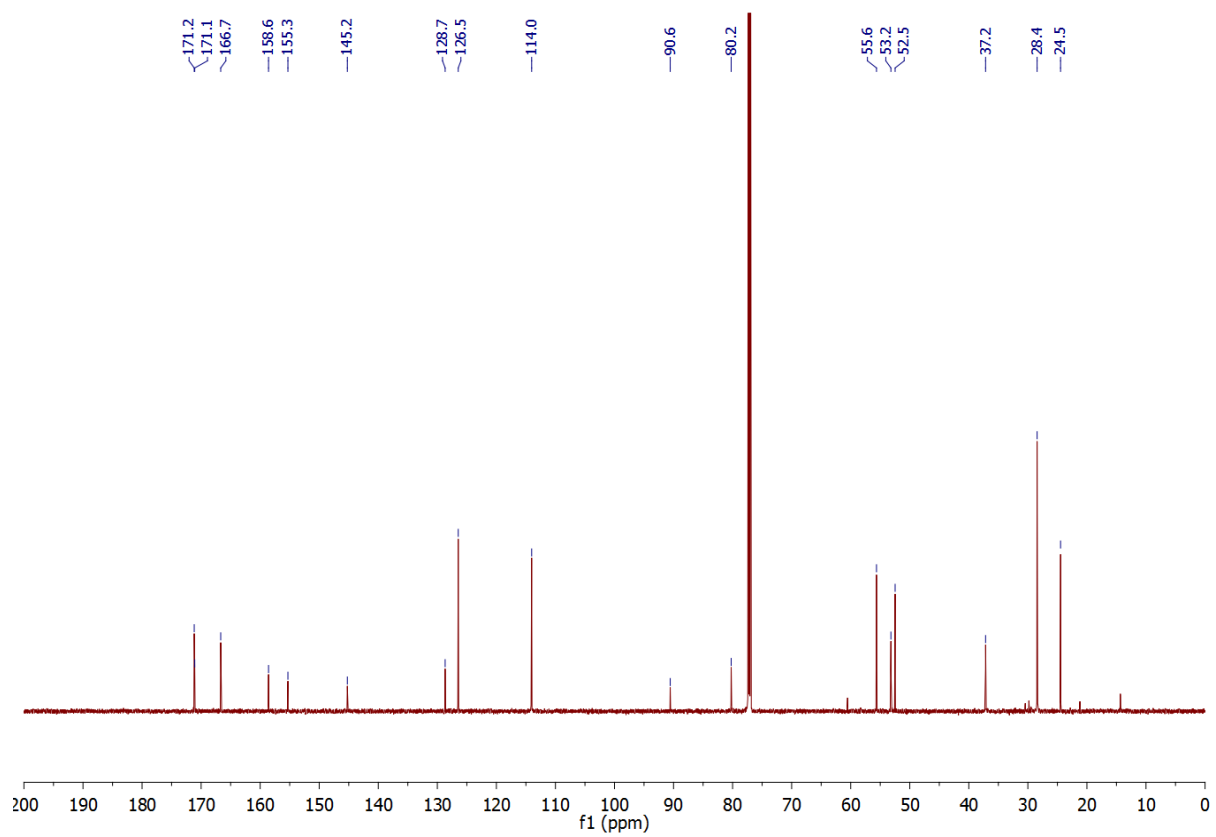
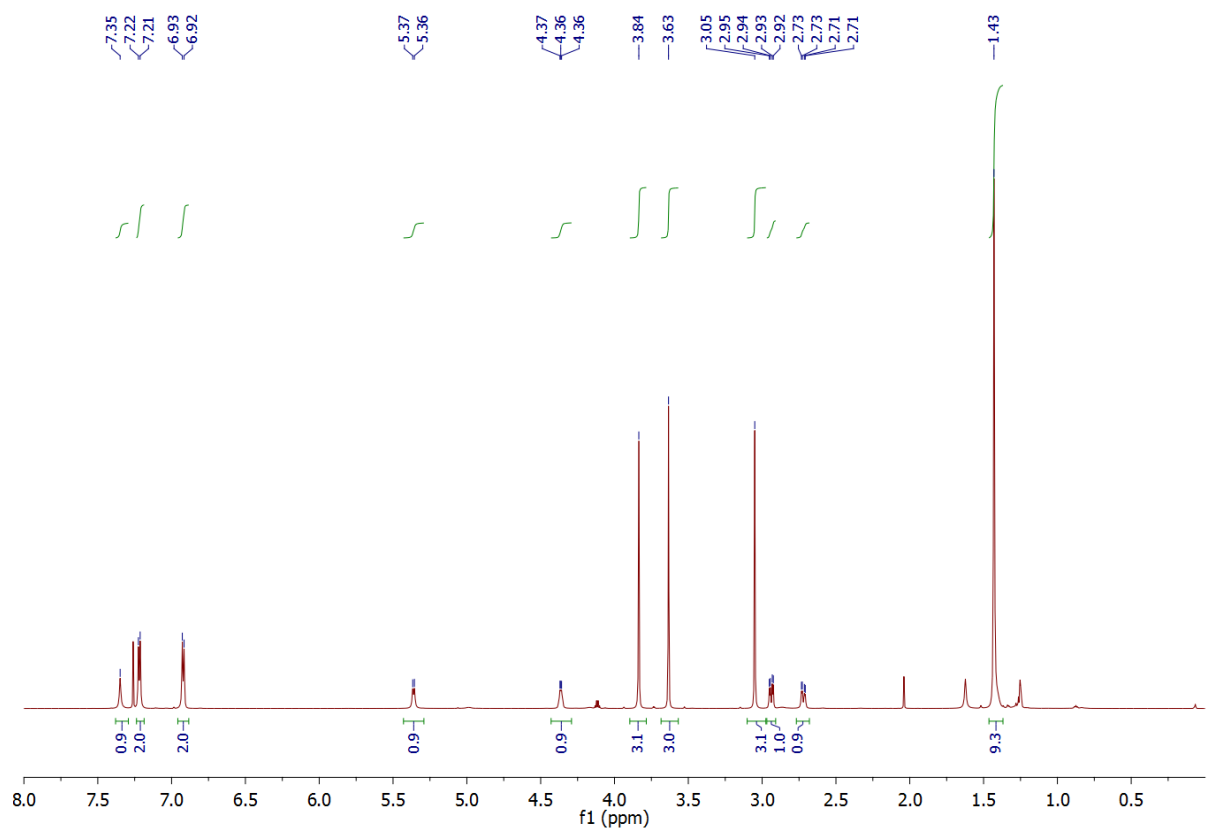


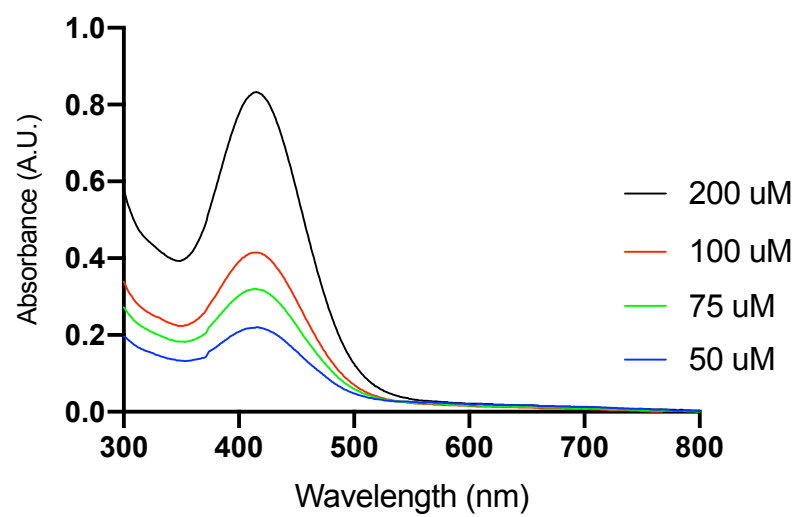
Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(4-((4-methoxyphenyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**11**)



To methyl S - (4 - bromo - 1 - methyl - 2,5 - dioxo - 2,5 - dihydro - 1*H* - pyrrol - 3 - yl) - *N* - (*tert* - butoxycarbonyl) - *L* - cysteinate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL), *p*-anisidine (0.012 g, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 24 hours at room temperature. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30mL) and washed with 10% citric acid solution (2 x 10 mL), water (1 x 10 mL) and dried over MgSO₄. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-50% EtOAc /hexane) yielded the product as a yellow oil (0.021 g, 0.045 mmol, 90%);

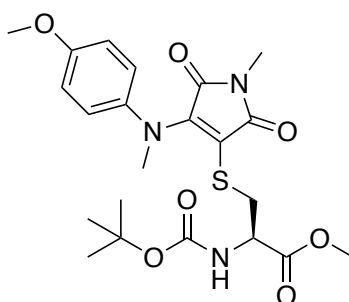
¹H NMR (700 MHz, CDCl₃); δ 7.35 (s, 1H, ArNH), 7.22 (d, *J* = 8.3 Hz, 2H, ArH), 6.92 (d, *J* = 8.4 Hz, 2H, ArH), 5.36 (d, *J* = 8.4 Hz, 1H, NH), 4.36 (dt, *J* = 9.4, 5.0 Hz, 1H, CH), 3.84 (s, 3H, ArOCH₃), 3.63 (d, *J* = 1.1 Hz, 3H, COOCH₃), 3.05 (s, 3H, NCH₃), 2.94 (dd, *J* = 14.1, 5.0 Hz, 1H, SCHH), 2.72 (dd, *J* = 14.1, 4.7 Hz, 1H, SCHH), 1.43 (s, 9H, (CH₃)₃); ¹³C NMR (176 MHz, CDCl₃); δ 171.2 (CO), 171.1 (CO), 166.7 (CO), 158.6 (CO), 155.3 (C), 145.2 (C), 128.7 (C), 126.5 (C), 114.0 (C), 90.6 (SC), 80.2 (C), 55.6 (CH), 53.2 (CH₃), 52.5 (CH₃), 37.2 (CH₂), 28.4 (CH₃), 24.5 (CH₃); IR (thin film); 1679 (s), 1703 (s), 1752 (s), 3340 (m), 3374 (m) cm⁻¹; LRMS (ES⁺) 466.2 (100, [M+H]⁺); HRMS (ES⁺) calcd for C₂₁H₂₈N₃O₇S [M+H]⁺ 466.1648, observed 466.1632. ε₄₁₅ 4250 M⁻¹.





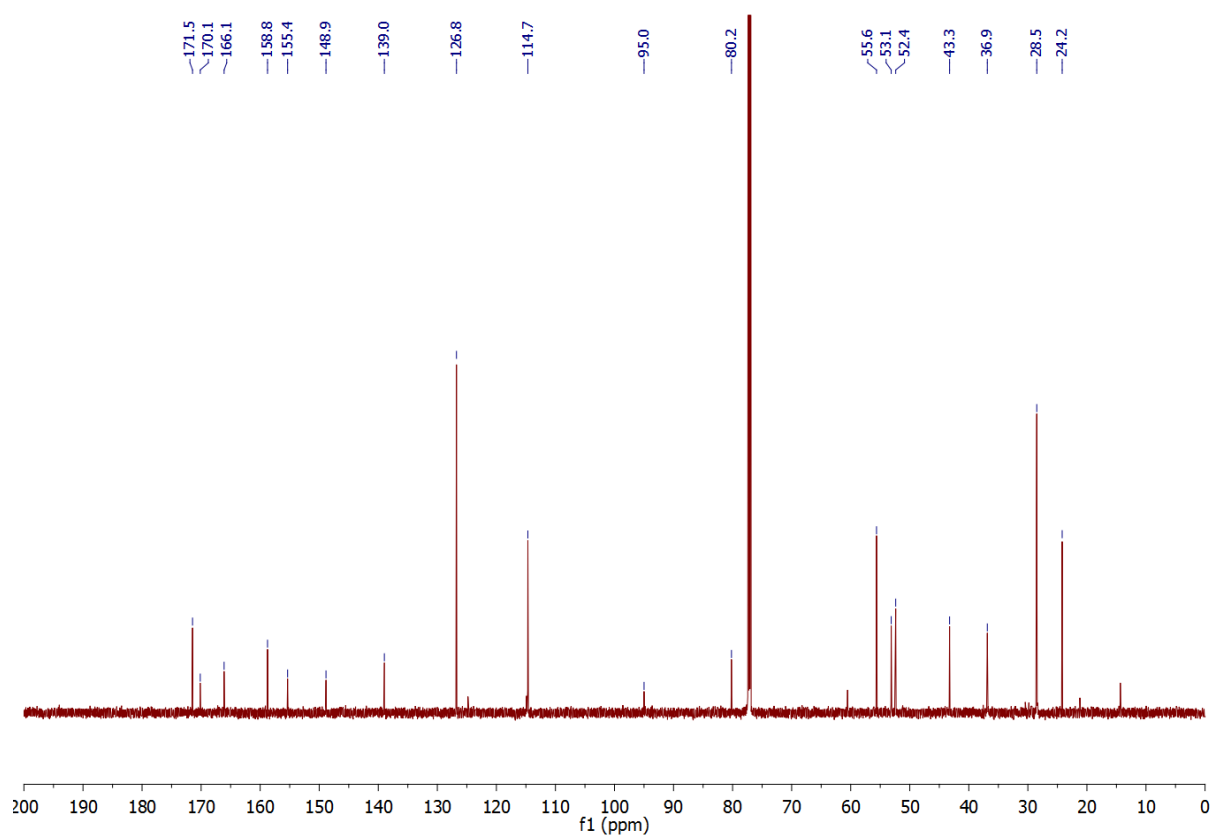
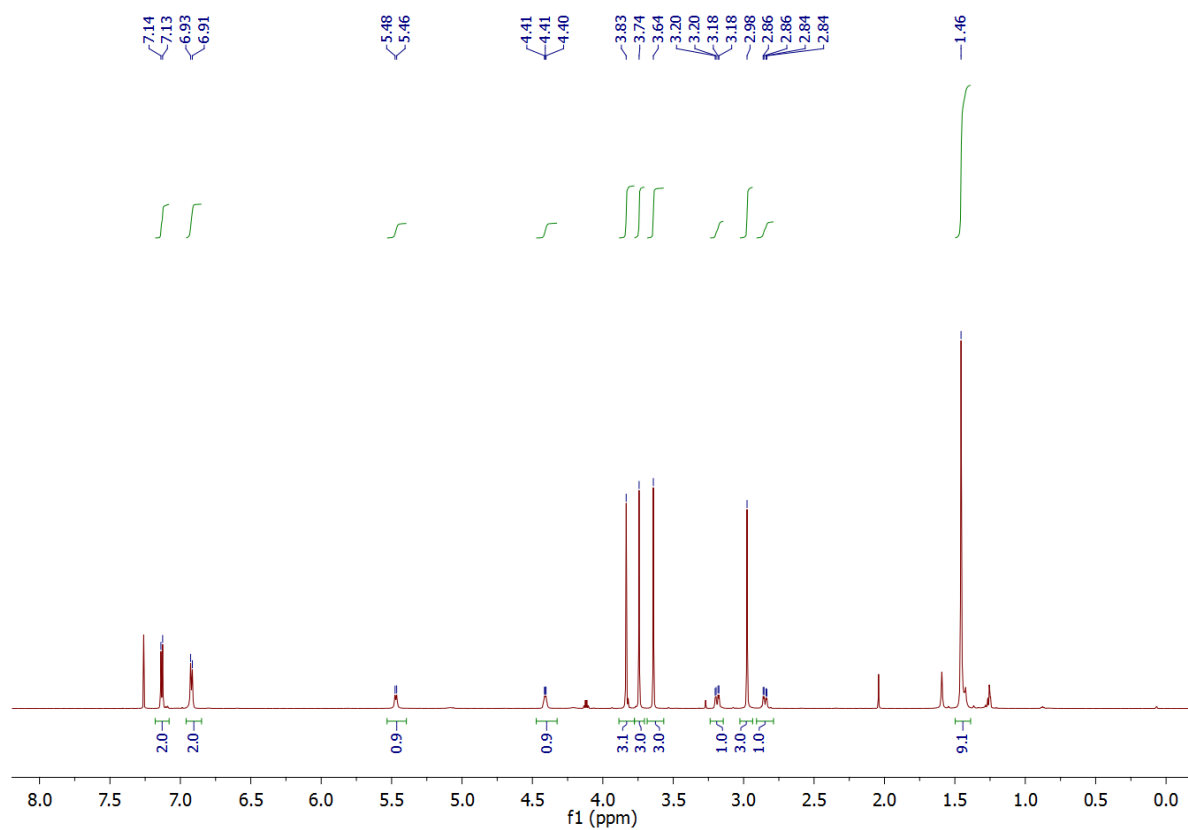
Absorbance spectra recorded in 10% THF in PBS (pH 7.4).

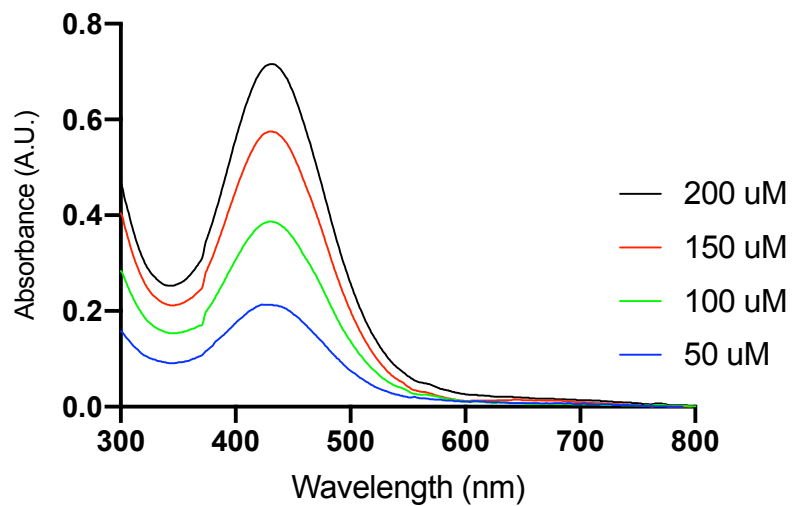
Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(4-((4-methoxyphenyl)(methyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**12**)



To methyl S - (4 - bromo - 1 - methyl - 2,5 - dioxo - 2,5 - dihydro - 1*H* - pyrrol - 3 - yl) - *N* - (*tert* - butoxycarbonyl) - *L* - cysteinate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL), *N*-methyl *p*-anisidine (0.014 g, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 24 hours at room temperature. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30 mL) and washed with 10% citric acid solution (2 x 10 mL), water (1 x 10 mL) and dried over MgSO₄. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-50% EtOAc/hexane) yielded the product as an orange oil (0.021 g, 0.044 mmol, 88%);

¹H NMR (700 MHz, CDCl₃); δ 7.13 (d, *J* = 8.4 Hz, 2H, ArH), 6.92 (d, *J* = 8.4 Hz, 2H, ArH), 5.47 (d, *J* = 8.4 Hz, 1H, NH), 4.41 (dt, *J* = 9.2, 4.8 Hz, 1H, CH), 3.83 (s, 3H, ArOCH₃), 3.74 (s, 3H, ArNCH₃), 3.64 (s, 3H, COOCH₃), 3.19 (dd, *J* = 14.1, 4.9 Hz, 1H, SCHH), 2.98 (s, 3H, NCH₃), 2.85 (dd, *J* = 14.2, 4.7 Hz, 1H, SCHH), 1.46 (s, 9H, (CH₃)₃); ¹³C NMR (176 MHz, CDCl₃) δ 171.5 (CO), 170.1 (CO), 166.1 (CO), 158.8 (CO), 155.4 (C), 148.9 (C), 139.0 (C), 126.8 (C), 114.7 (C), 95.0 (SC), 80.2 (C), 55.6 (CH), 53.1 (CH₃), 52.4 (CH₃), 43.3 (CH₃), 36.9 (CH₂), 28.5 (CH₃), 24.2 (CH₃); IR (thin film); 1697 (s), 1747 (s), 2932 (br), 2999 (w), 3366 (m) cm⁻¹; LRMS (ES⁺) 480.2 (100, [M+H]⁺); HRMS (ES⁺) calcd for C₂₂H₃₀N₃O₇S [M+H]⁺ 480.1804, observed 480.1798.





Absorbance spectra recorded in 10% THF in PBS (pH 7.4).

Amine Reactions in Buffer

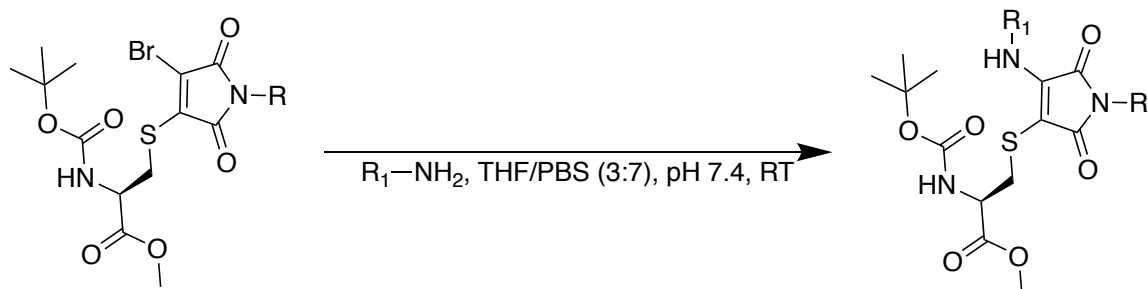
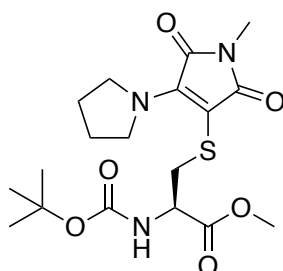


Table S2: Amine reactions in THF/PBS buffer.

R	R ¹ -(Amine)	Equiv. of amine	Yield
Me	Pyrrolidine	100	72%
Me	Aniline	100	96%
Me	<i>p</i> -anisidine	10	90%
Me	<i>p</i> -anisidine	100	86%
Me	<i>N</i> -Me <i>p</i> -anisidine	10	76%

All reactions are 21 mg **6** in 50 mL (1 mM) final volume. PBS (pH 7.4, 25 mM phosphates) was used.

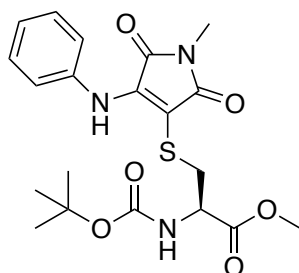
Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(2,5-dioxo-1-methyl-4-(pyrrolidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (**9**)



Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (15 mL). Pyrrolidine (0.411 mL, 5.000 mmol) was dissolved in phosphate buffer solution (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature for 1 hour. The solution was extracted with CHCl₃ (3 × 30 mL) and dried over MgSO₄. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.015 g, 0.036 mmol, 72%).

Analytical data matches that of the organic solvent method to compound (vide supra).

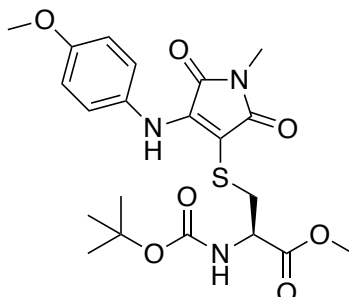
Synthesis of methyl *N*-(tert-butoxycarbonyl)-*S*-(1-methyl-2,5-dioxo-4-(phenylamino)-2,5-dihydro-1H-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (**10**)



Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-*N*-(tert-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (5 mL). Aniline (0.466 g, 0.457 μ L, 5.000 mmol) in THF (10 mL) was dissolved in phosphate buffer solution (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature overnight. Brine (10 mL) was added to the solution, and the organic product was extracted with CHCl_3 (3 \times 30 mL) and dried over MgSO_4 . The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.021 g, 0.048 mmol, 96%).

Analytical data matches that of the organic solvent method to compound (vide supra).

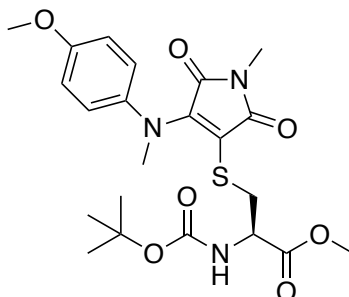
Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(4-((4-methoxyphenyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (11)



Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (5 mL). *p*-anisidine (0.062 g, 0.500 mmol) in THF (10 mL) was dissolved in phosphate buffer solution (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature for 1 hour. Brine (10 mL) was added to the solution, and the organic product was extracted with CHCl₃ (3 × 30 mL) and dried over MgSO₄. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30 mL) and washed with 10% citric acid solution (2 × 10 mL), water (1 × 10 mL) and dried over MgSO₄. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-50% EtOAc /hexane) yielded the product as a yellow oil (0.021 g, 0.045 mmol, 90%). *N.B. Same protocol followed for 100 equiv. experiment shown in table S2.*

Analytical data matches that of the organic solvent method to compound (vide supra).

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(4-((4-methoxyphenyl)(methyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (**12**)

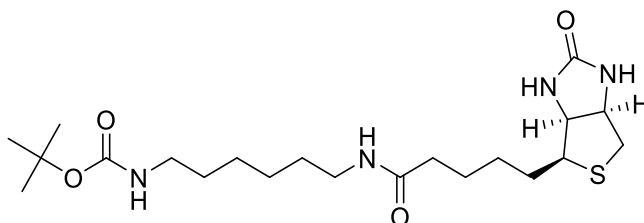


Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (5 mL). *N*-methyl *p*-anisidine (0.067 g, 0.500 mmol) in THF (10 mL) was dissolved in phosphate buffer solution (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature for 1 hour. Brine (10 mL) was added to the solution, and the organic product was extracted with CHCl₃ (3 × 30 mL) and dried over MgSO₄. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30 mL) and washed with 10% citric acid solution (2 × 10 mL), water (1 × 10 mL) and dried over MgSO₄. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-40% EtOAc/hexane) yielded the product as an orange oil (0.018 g, 0.038 mmol, 76%).

Analytical data matches that of the organic solvent method to compound (vide supra).

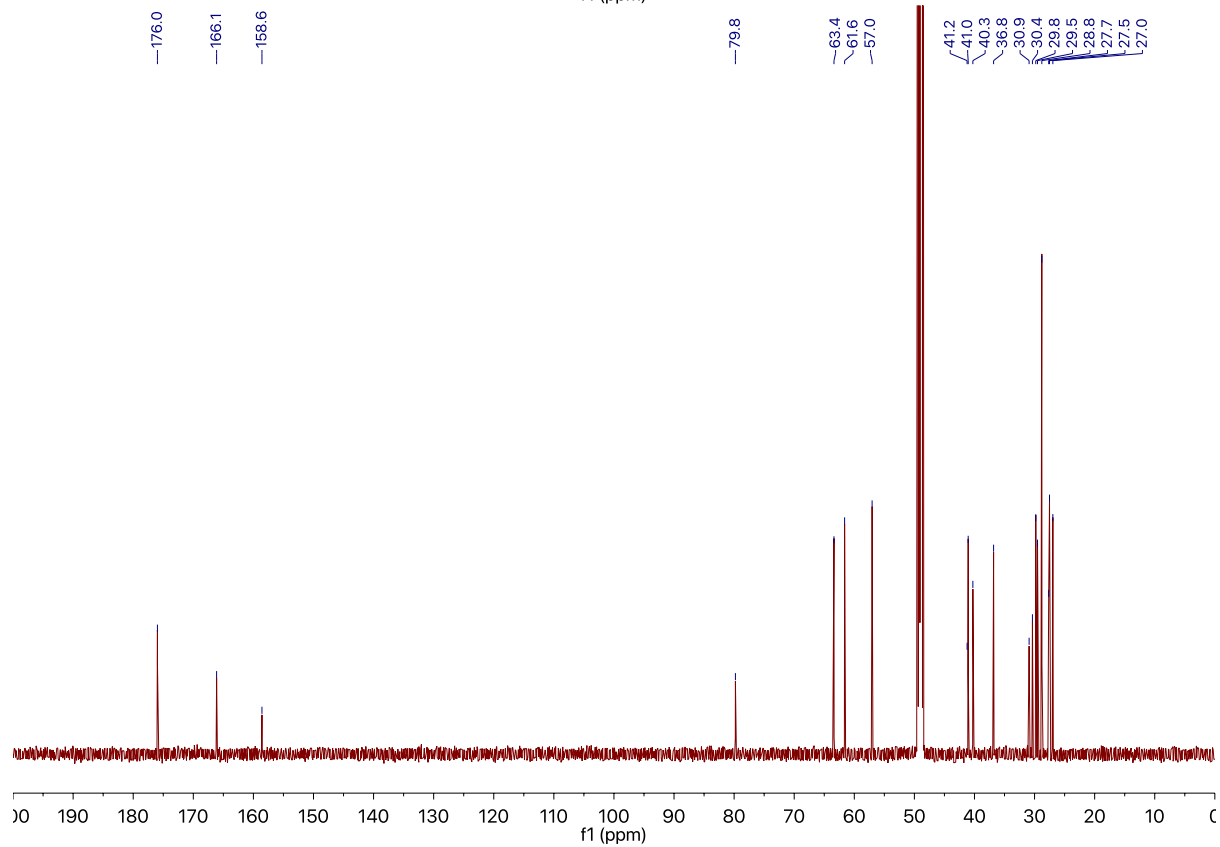
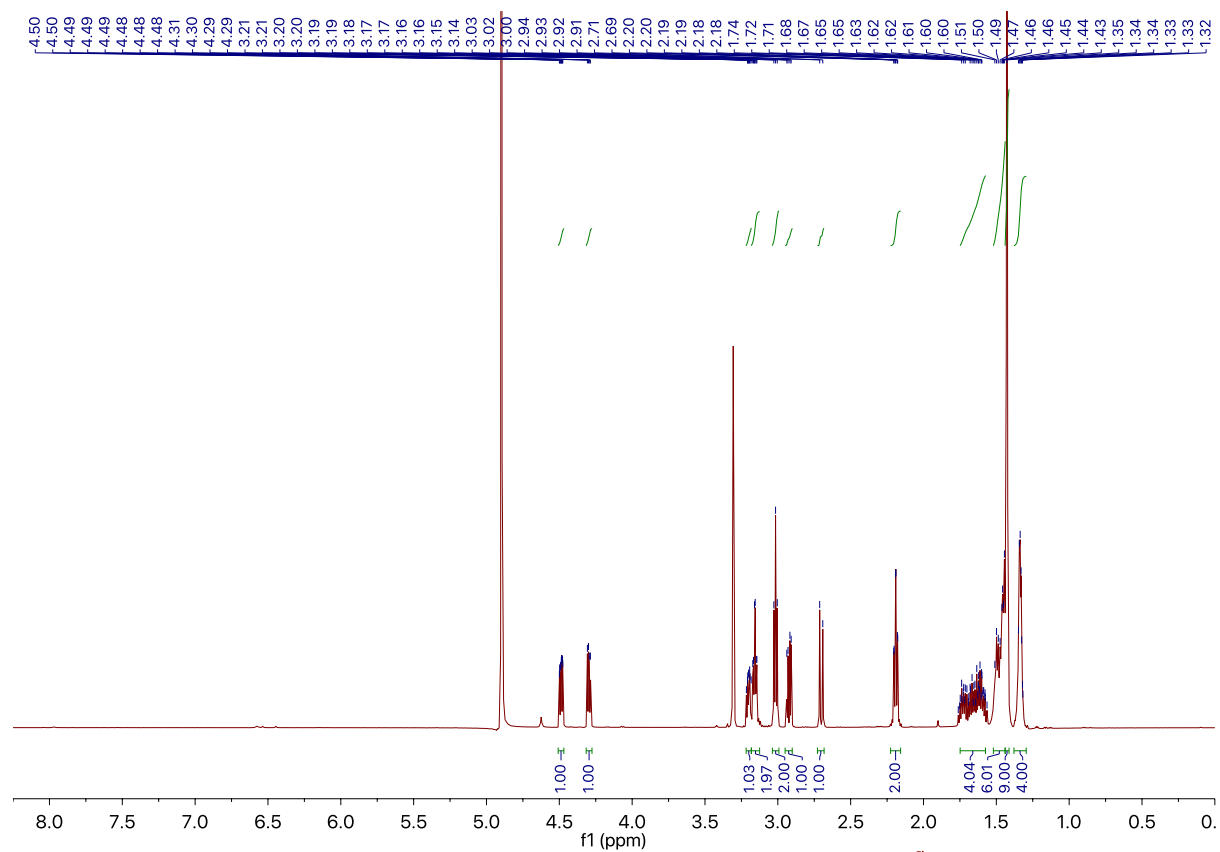
Synthesis of functionalised DBMs

Synthesis of tert-butyl (6-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido) hexyl)carbamate (**29**)

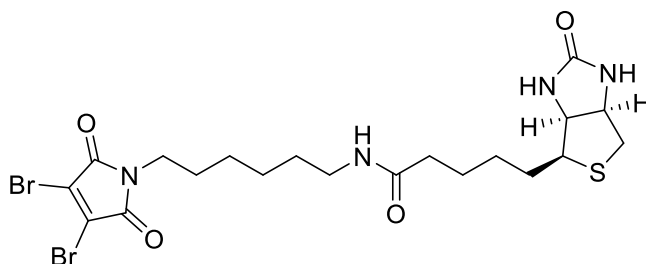


D-Biotin (221 mg, 0.905 mmol), HBTU (360 mg, 0.949 mmol) and DIPEA (236 μ L, 1.36 mmol) were dissolved in DMF (5 mL) and stirred for 20 min at RT. *N*-Boc-1,6-hexanediamine (205 mg, 1.16 mmol) in DMF (2 mL) was then added and the resultant mixture was stirred for 24 h at RT. After this period, the solvent was removed *in vacuo*. Purification by column chromatography (0-10% MeOH/DCM) afforded the target compound as a white crystalline solid (396 mg, 0.895 mmol, 99%).

m.p. 184.0-186.0 $^{\circ}$ C; ^1H NMR (600 MHz, CD_3OD); δ 4.49 (dd, $J = 7.8, 4.6$ Hz, 1H, SCH_2CH), 4.30 (dd, $J = 7.9, 4.5$ Hz, 1H, SCHCH), 3.22-3.18 (m, 1H, SCH), 3.16 (td, $J = 6.9, 2.8$ Hz, 2H, $\text{CH}_2\text{NHC}(\text{O})\text{CH}_2$), 3.02 (t, $J = 7.0$ Hz, 2H, $\text{OC}(\text{O})\text{NHCH}_2$), 2.92 (dd, $J = 12.7, 5.0$ Hz, 1H, SCHH), 2.70 (d, $J = 12.7$ Hz, 1H, SCHH), 2.19 (t, $J = 7.2$ Hz, 2H, $\text{NHC}(\text{O})\text{CH}_2$), 1.76-1.56 (m, 4H, SCHCH_2 and $\text{SCHCH}_2\text{CH}_2\text{CH}_2$), 1.52-1.44 (m, 6H, $\text{OC}(\text{O})\text{NHCH}_2\text{CH}_2$, $\text{CH}_2\text{C}(\text{O})\text{NHCH}_2\text{CH}_2$ and $\text{SCHCH}_2\text{CH}_2$), 1.43 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.35-1.32 (m, 4H, $\text{OC}(\text{O})\text{NHCH}_2\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{C}(\text{O})\text{NHCH}_2\text{CH}_2\text{CH}_2$); ^{13}C NMR (150 MHz, CD_3OD); δ 176.0 (CO), 166.1 (CO), 158.6 (CO), 79.8 (C), 63.4 (CH), 61.6 (CH), 57.0 (CH), 41.2 (CH_2), 41.0 (CH_2), 40.2 (CH_2), 36.8 (CH_2), 30.9 (CH_2), 30.4 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 28.8 (CH_3), 27.7 (CH_2), 27.5 (CH_2), 27.0 (CH_2); IR (solid); 1516 (s), 1621 (m), 1687 (s), 2932 (w), 3252 (w, br), 3386 (w) cm^{-1} ; LRMS (ESI) m/z 465.2 ($[\text{M}+\text{Na}]^+$, 17), 443.3 ($[\text{M}+\text{H}]^+$, 100), 343.2 (45); HRMS (ESI) calcd for $\text{C}_{21}\text{H}_{39}\text{N}_4\text{OS}$ $[\text{M}+\text{H}]^+$ 443.2687, observed 443.2675.

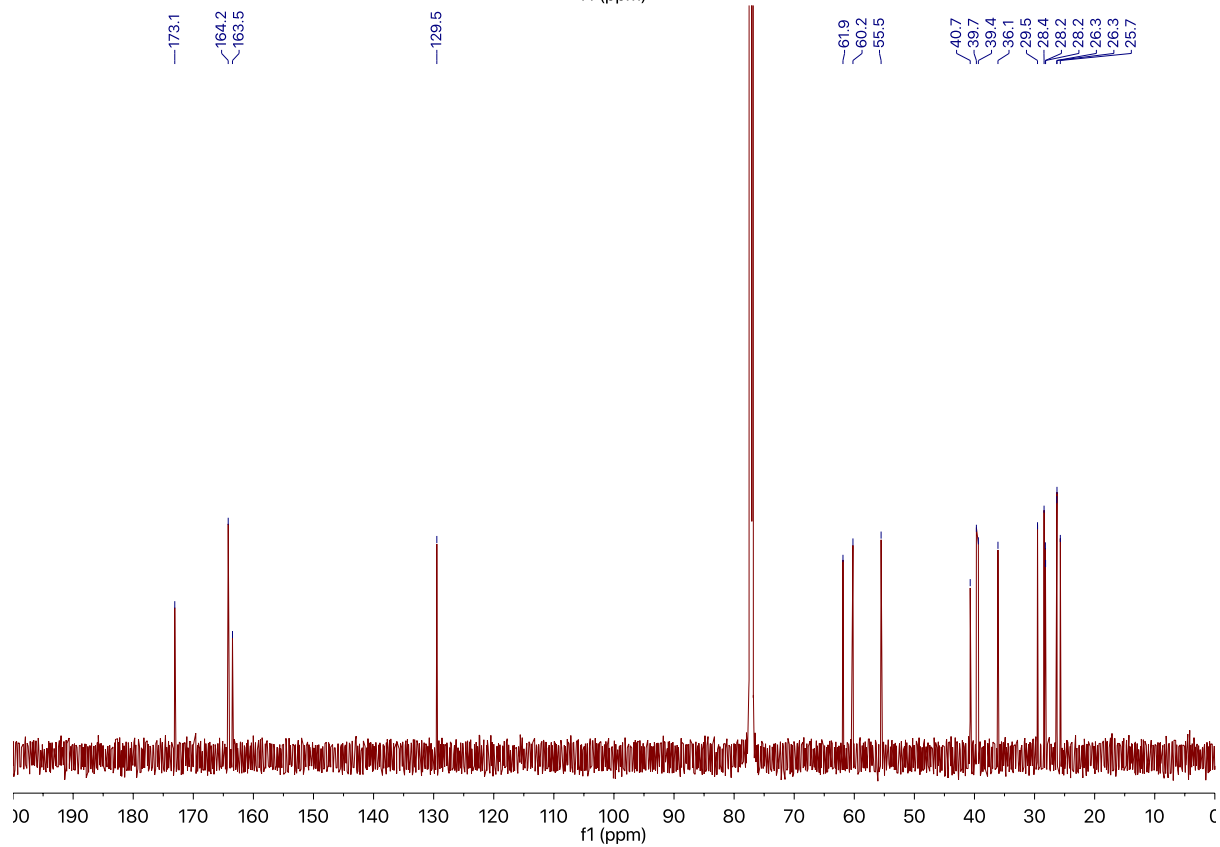
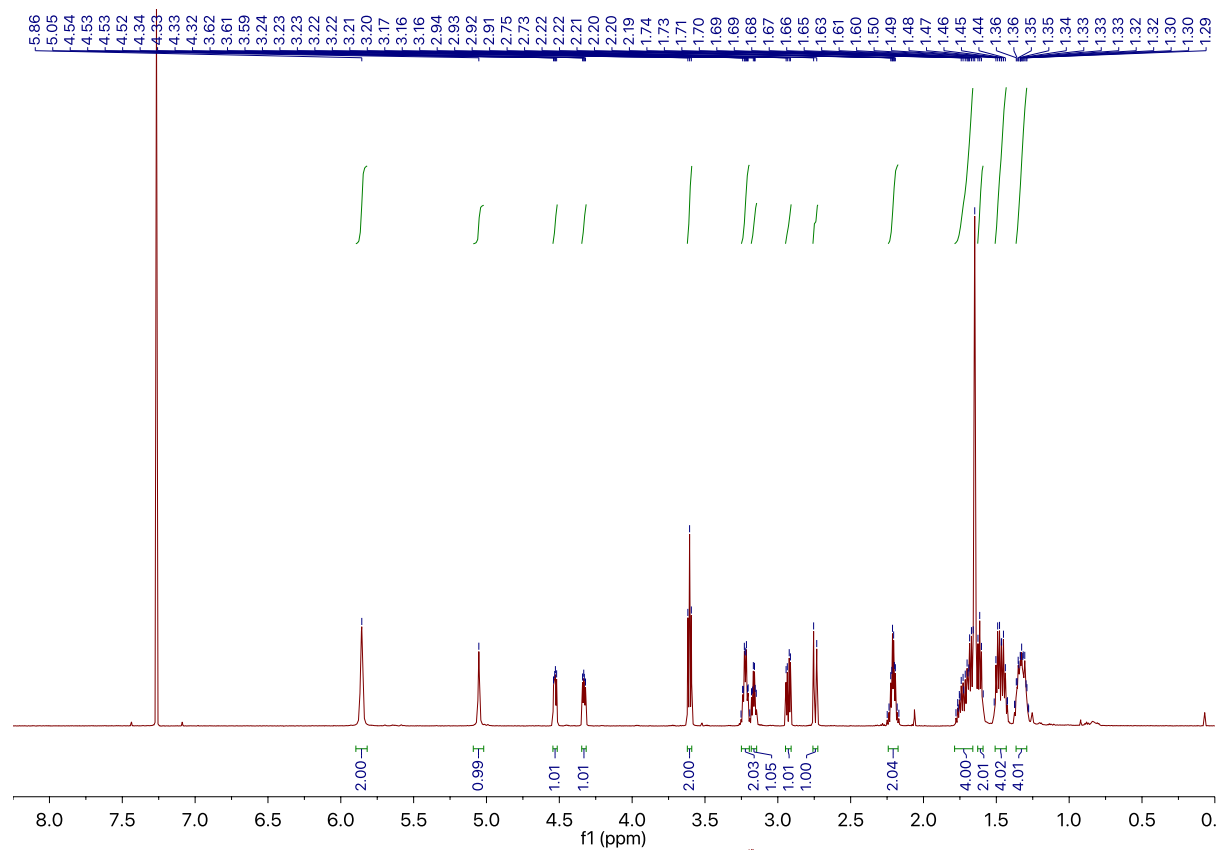


Synthesis of *N*-(6-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexyl)-5-((3*a*S,4*S*,6*a*R)-2-oxohexahydro-1H-thieno[3,4-*d*]imidazol-4-yl)pentanamide (**21**)



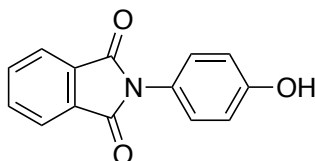
Compound **29** (180 mg, 0.407 mmol) was dissolved in TFA (8 mL). After 18 h at RT, the solvent was removed *in vacuo*. The crude residue was dissolved in AcOH (18 mL) and dibromomaleic acid (112 mg, 0.409 mmol) was added. The reaction was heated to reflux for 3 h. All volatile material was then removed *in vacuo*. Purification by column chromatography (0-12% MeOH/DCM) afforded the product as a pale yellow solid (143 mg, 0.247 mmol, 61%).

m.p. 158.0-160.0 °C; ^1H NMR (600 MHz, CD_3Cl); δ 5.86 (br s, 2H, NHC(O)NH), 5.05 (br s, 1H, NHC(O)), 4.53 (dd, $J = 7.5, 5.1$ Hz, 1H, SCH_2CH), 4.33 (dd, $J = 7.3, 4.9$ Hz, 1H, SCHCH), 3.61 (t, $J = 7.2$ Hz, 2H, NCH_2), 3.25-3.19 (m, 2H, NHCH_2), 3.18-3.15 (m, 1H, SCH), 2.93 (dd, $J = 12.9, 5.0$ Hz, 1H, SCHH), 2.74 (d, $J = 12.8$ Hz, 1H, SCHH), 2.25-2.17 (m, 2H, NHC(O)CH_2), 1.78-1.66 (m, 4H, SCHCH_2 and $\text{C(O)CH}_2\text{CH}_2$), 1.63-1.59 (m, 2H, NCH_2CH_2), 1.51-1.42 (m, 4H, NHCH_2CH_2 and $\text{SCHCH}_2\text{CH}_2$), 1.37-1.28 (m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2$ and $\text{NHCH}_2\text{CH}_2\text{CH}_2$); ^{13}C NMR (150 MHz, CDCl_3); δ 173.1 (CO), 164.2 (CO), 163.5 (CO), 129.5 (CBr), 61.9 (CH), 60.2 (CH), 55.5 (CH), 40.7 (CH_2), 39.7 (CH_2), 39.4 (CH_2), 36.1 (CH_2), 29.5 (CH_2), 28.4 (CH_2), 28.2 (CH_2), 28.2 (CH_2), 26.3 (CH_2), 26.3 (CH_2), 25.7 (CH_2); IR (solid); 1544 (m), 1641 (m), 1693 (s), 1715 (s), 2859 (w), 2931 (w), 3289 (w, br) cm^{-1} ; LRMS (ESI) m/z 605 ($[\text{}^{81,81}\text{M}+\text{Na}]^+$, 8), 603 ($[\text{}^{81,79}\text{M}+\text{Na}]^+$, 16), 601 ($[\text{}^{79,79}\text{M}+\text{Na}]^+$, 8), 583 ($[\text{}^{81,81}\text{M}+\text{H}]^+$, 54), 581 ($[\text{}^{81,79}\text{M}+\text{H}]^+$, 100), 579 ($[\text{}^{79,79}\text{M}+\text{Na}]^+$, 49); HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{29}^{81,79}\text{Br}_2\text{N}_4\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 581.0251, observed 581.0242.



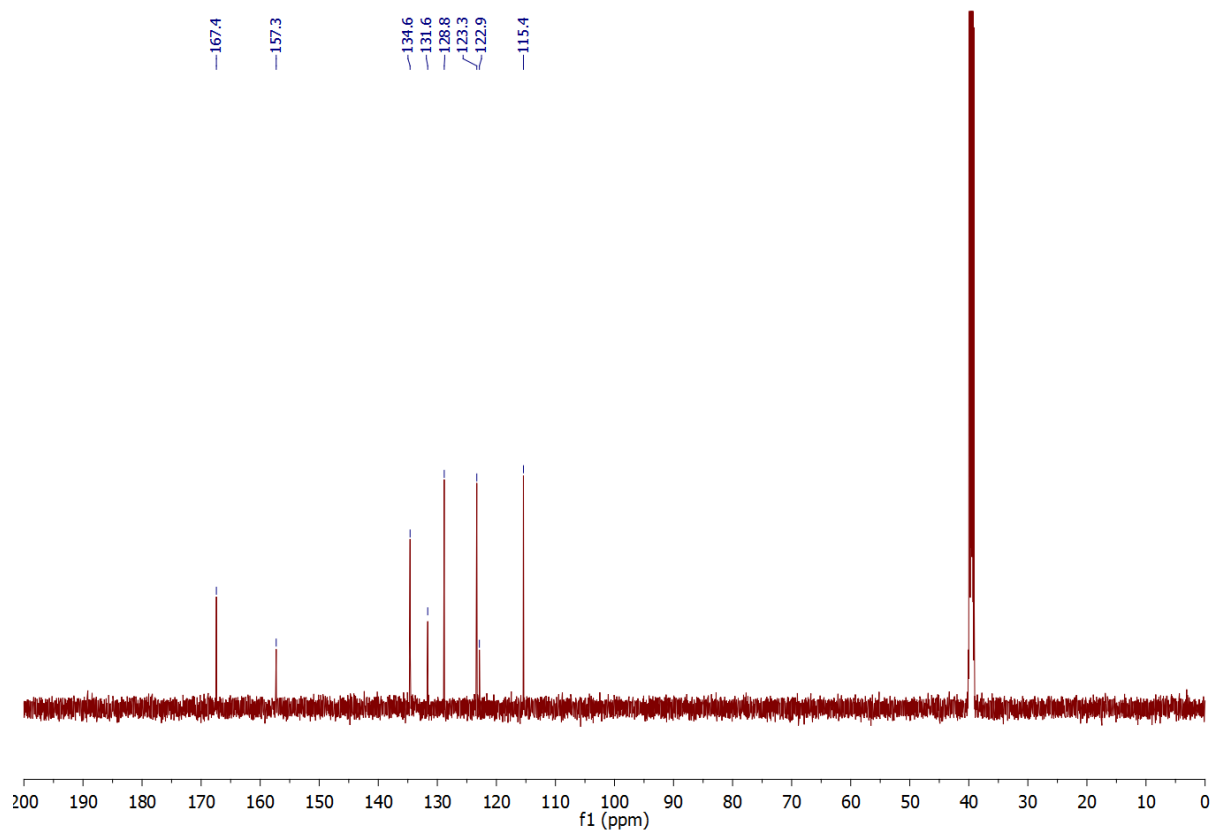
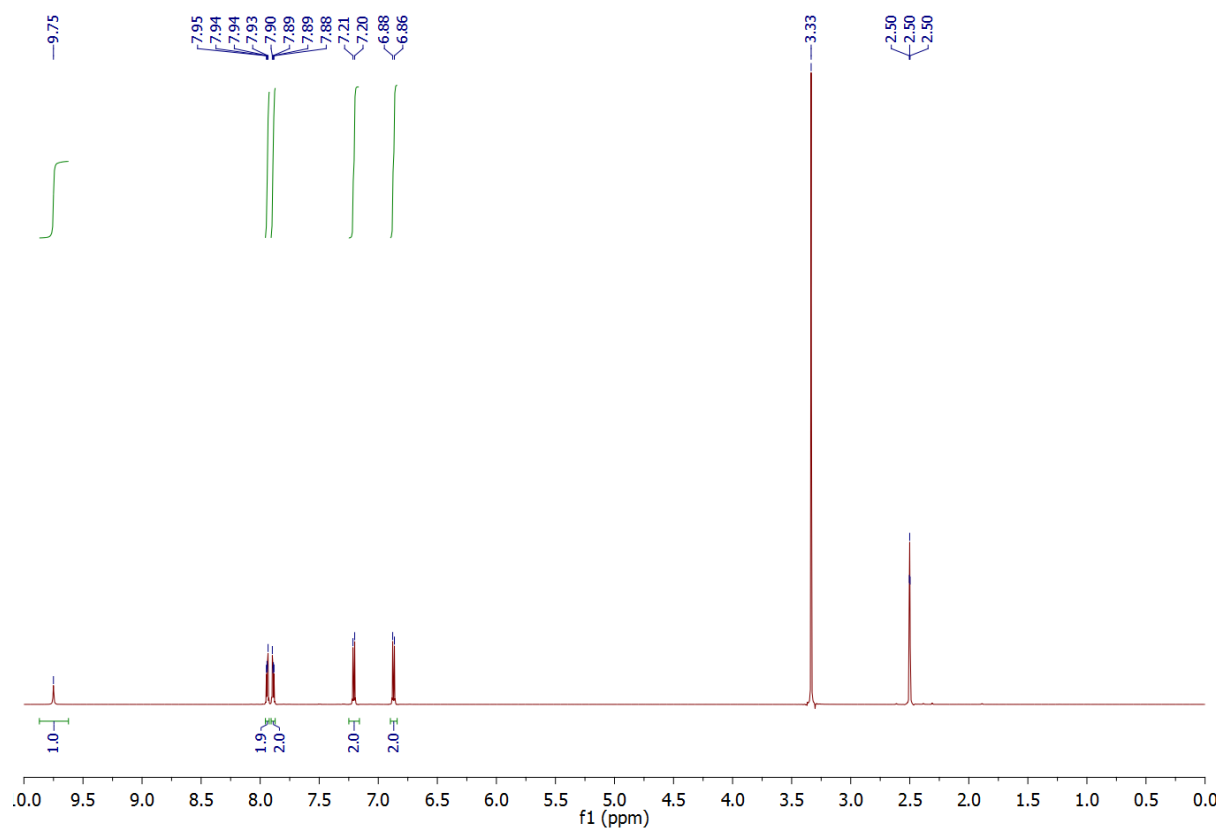
Synthesis of *p*-Anisidine Analogues

Synthesis of 2-(4-hydroxyphenyl)isoindoline-1,3-dione⁴ (**30**)

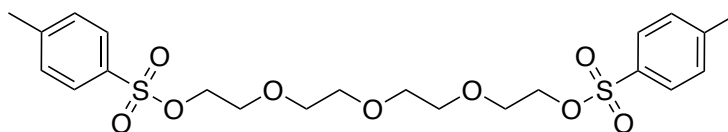


Synthesised according to reference. *p*-Aminophenol (0.275 g, 2.52 mmol) was added to phthalic anhydride (0.373 g, 2.52 mmol) dissolved in acetic acid (10 mL). The mixture was stirred and refluxed for 16 h. After cooling to room temperature, the precipitate was filtered and washed with methanol (10 mL). Removal of solvent by evaporation afforded a violet solid (0.391 g, 1.63 mmol, 65%).

¹H NMR (600 MHz, DMSO); δ 9.75 (s, 1H, OH), 7.95-7.93 (m, 2H, ArH), 7.90-7.88 (m, 2H, ArH), 7.21 (dt, J = 8.9, 2.8 Hz, 2H, ArH), 6.87 (dt, J = 8.9, 2.8 Hz, 2H, ArH); ¹³C NMR (151 MHz, DMSO); δ 167.4 (CO), 157.3 (ArC), 134.6 (ArC), 131.6 (ArC), 128.8 (ArC), 123.3 (ArC), 122.9 (ArC), 115.4 (ArC).

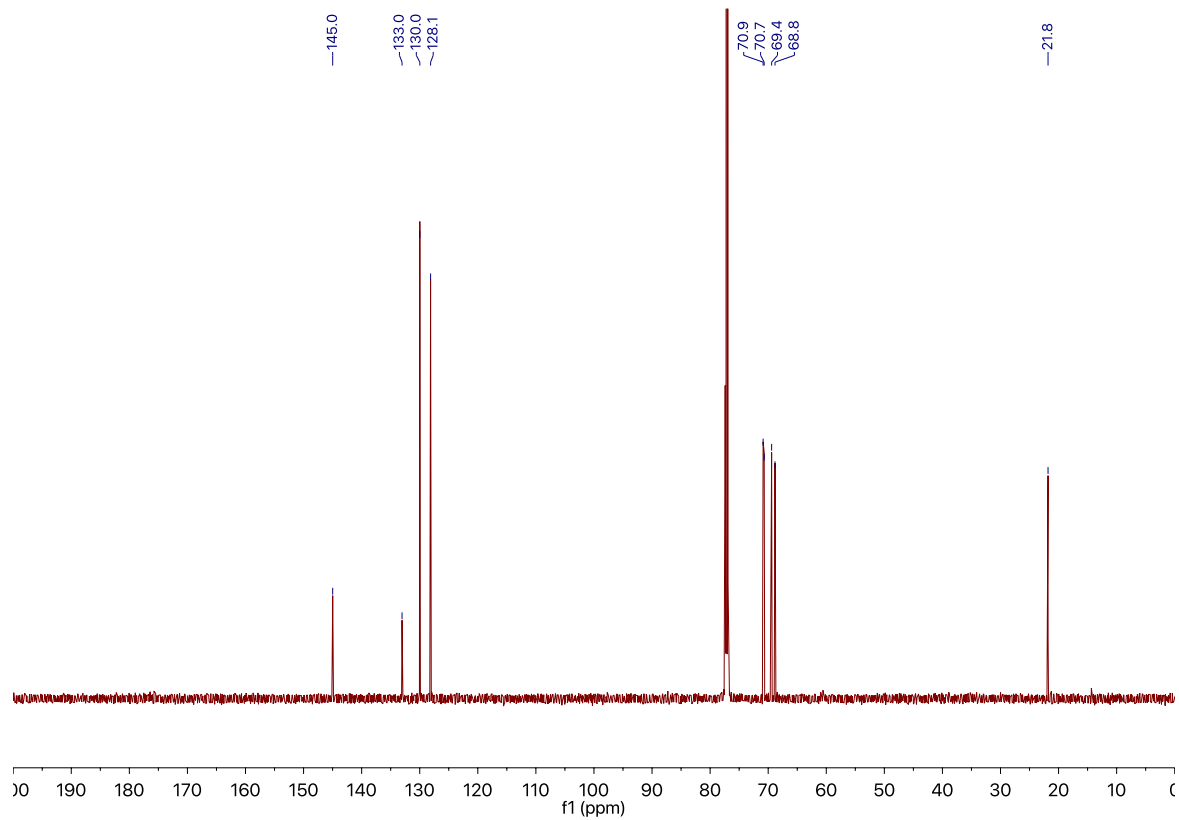
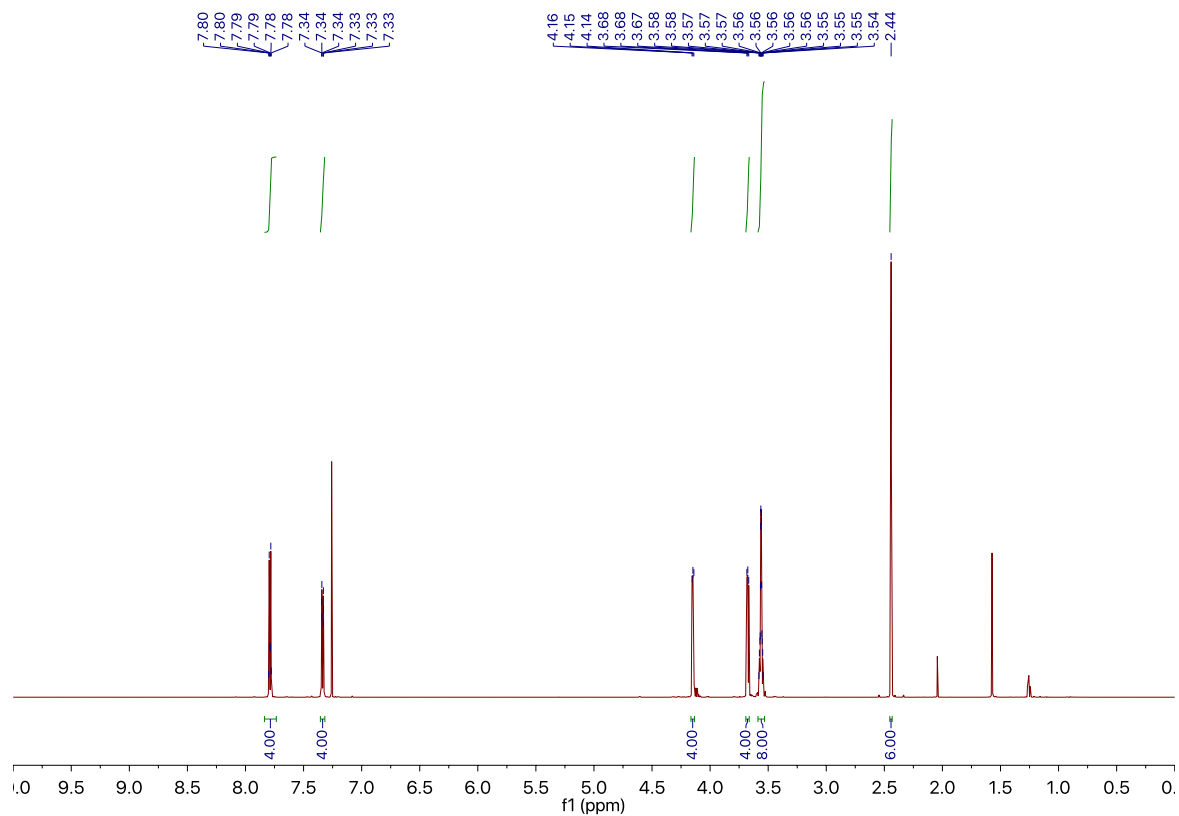


Synthesis of ((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate)⁵ (**31**)

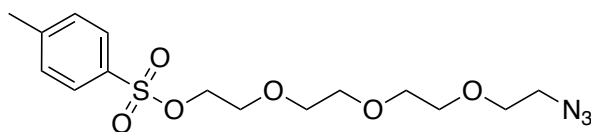


Tosyl chloride (2.33 g, 12.23 mmol) dissolved in DCM (5 mL) was added in two portions, 30 min apart, to a stirred solution of tetra ethylene glycol (1.08 g, 5.56 mmol) in pyridine (3.6 mL) at 0 °C. The mixture was stirred for 6 h at 0 °C. Ice water (30 mL) was added to the reaction mixture and the target molecule was extracted with EtOAc (3 x 30 mL). The organic layer was washed with HCl (2M, 2 x 13 mL). The organic layer was then dried with MgSO₄, filtered and the solvent removed on the rotary evaporator to afford a colourless oil (1.74 g, 3.46 mmol, 62%).

¹H NMR (600 MHz, CDCl₃); δ 7.79 (dt, *J* = 8.4, 2.0 Hz, 4H, ArH), 7.34-7.33 (m, 4H, ArH), 4.16-4.14 (m, 4H, CH₂OS), 3.68-3.67 (m, 4H, OCH₂), 3.58-3.54 (m, 8H, 4 x OCH₂), 2.44 (s, 6H, 2 x CH₃); ¹³C NMR (151 MHz, CDCl₃); δ 145.0 (ArC), 133.0 (ArC), 130.0 (ArC), 128.1 (ArC), 70.9 (CH₂), 70.7 (CH₂), 69.4 (CH₂), 68.8 (CH₂), 21.8 (CH₃).

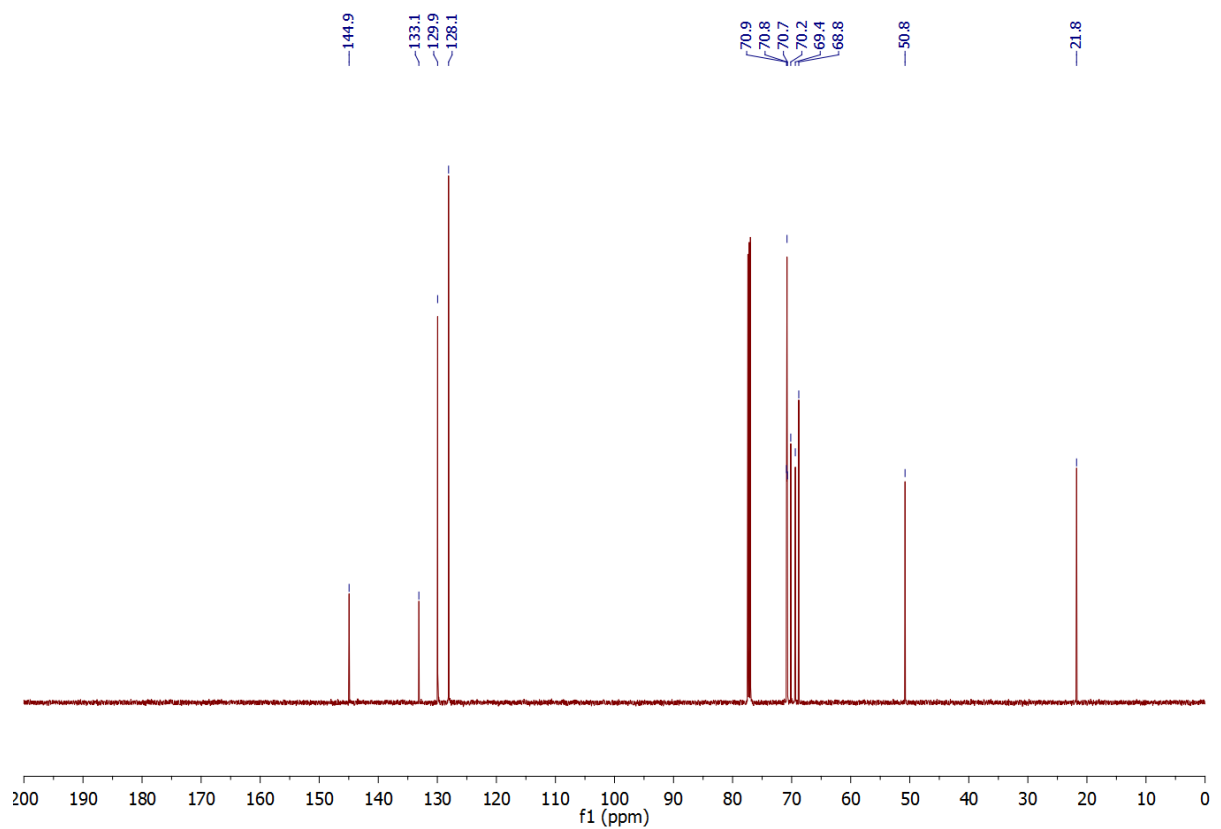


Synthesis of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate⁶
(32)

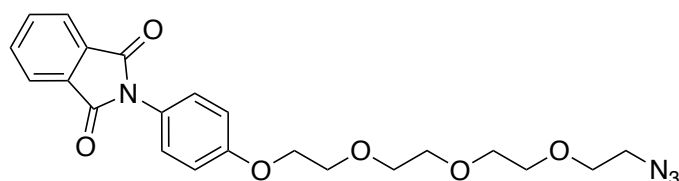


Synthesised according to reference. To a solution of tetraethylene glycol di-*para*-toluene sulfonate (0.240 g, 0.480 mmol) in ethanol (5 mL) was added sodium azide (0.033 g, 0.510 mmol). The resulting solution was heated at 80 °C for 16 h. The reaction was then poured into ice water (15 mL) and the product was extracted with ethyl acetate (3 x 15 mL). The organic extracts were washed with water (15 mL), brine (15 mL) and dried with MgSO₄. The resulting mixture was filtered, the solvent removed on the rotary evaporator and the crude product purified by column chromatography (25-80% EtOAc/petroleum ether) to yield a light brown oil (0.063 g, 0.169 mmol, 35%).

¹H NMR (600 MHz, CDCl₃); δ 7.79-7.76 (m, 2H, ArH), 7.33-7.32 (m, 2H, ArH), 4.15-4.13 (m, 2H, SOCH₂), 3.68-3.62 (m, 8H, OCH₂), 3.58-3.57 (m, 4H, OCH₂), 3.36 (t, 2H, *J* = 5.3 Hz, CH₂N₃), 2.43 (s, 3H, CH₃); ¹³C NMR (151 MHz, CDCl₃); δ 144.9 (ArC), 133.1 (ArC), 129.9 (ArC), 128.1 (ArC), 70.9 (CH₂), 70.8 (CH₂), 70.8 (CH₂), 70.7 (CH₂), 70.2 (CH₂), 69.4 (CH₂), 68.8 (CH₂), 50.8 (CH₂), 21.8 (CH₃); IR (thin film); 2100 (m), 2869 (w) cm⁻¹; LRMS (ES⁺) 391.2 (100, [M+NH₄]⁺); HRMS (ES⁺) calcd for C₁₅H₂₄N₃O₆S [M+NH₄]⁺ 391.1651, observed 391.1606.

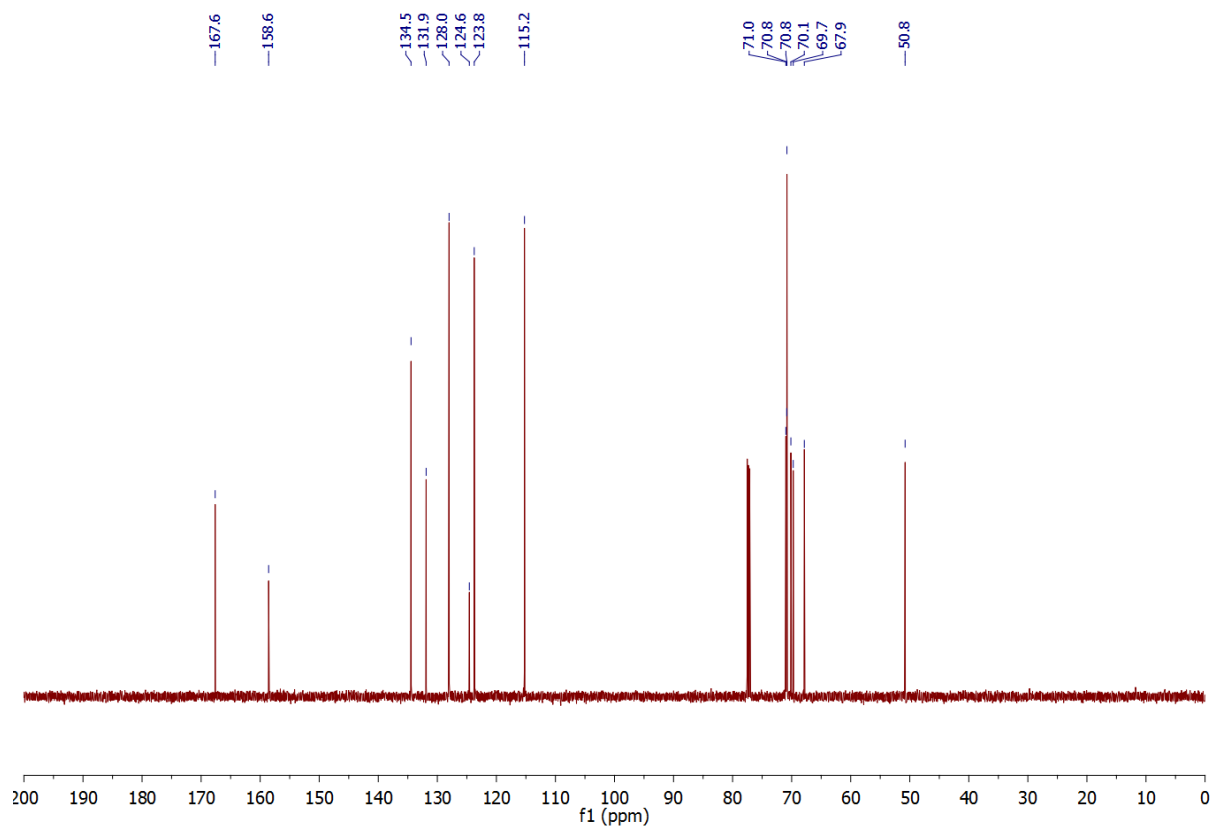
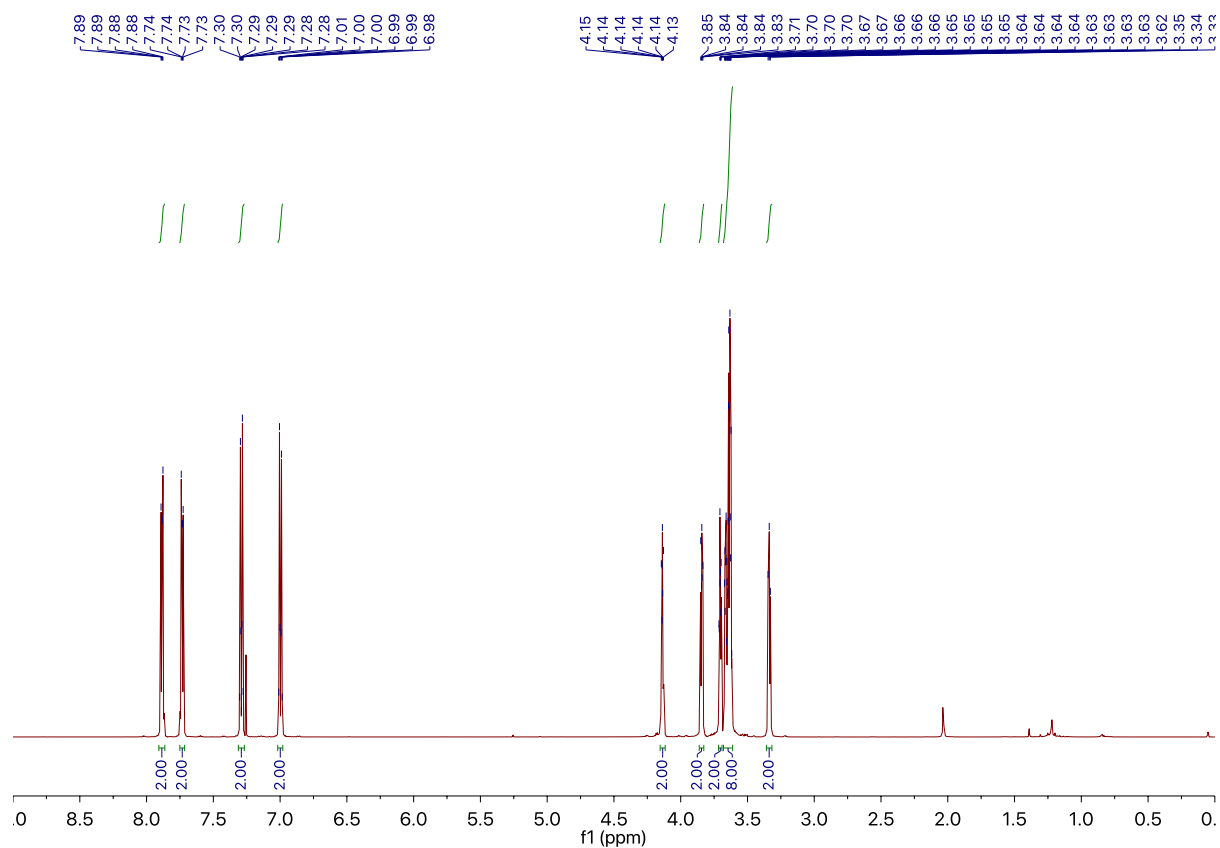


Synthesis of 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)isoindoline-1,3-dione (**33**)

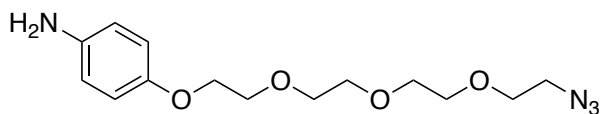


Phthalimide **30** (0.189 g, 0.791 mmol) was added to potassium *tert*-butoxide (725 μ L, 1M in THF) in DMF (20 mL) and left to stir at room temperature for 5 min. Azide **32** (0.245 g, 0.659 mmol) was then added, and the reaction mixture was heated at 90 °C for 16 h. Water (100 mL) was added to the reaction, and the organic phase was extracted with EtOAc (3 x 30 mL). The organic extracts were washed with saturated lithium chloride solution (2 x 30 mL) and then dried with MgSO₄. The solvent was removed on the rotary evaporator and the crude product was purified by column chromatography (25-80% EtOAc/petroleum ether). The product was collected as a clear yellow oil (0.202 g, 0.459 mmol, 70%).

¹H NMR (600 MHz, CDCl₃); δ 7.89-7.88 (m, 2H, ArH), 7.74-7.73 (m, 2H, ArH), 7.30-7.28 (m, 2H, ArH), 7.01-6.98 (m, 2H, ArH), 4.15-4.13 (m, 2H, OCH₂), 3.85-3.83 (m, 2H, OCH₂), 3.71-3.70 (m, 2H, OCH₂), 3.67-3.62 (m, 8H, 4 x OCH₂), 3.35-3.33 (m, 2H, CH₂N₃); ¹³C NMR (151 MHz, CDCl₃); δ 167.6 (CO), 158.6 (ArC), 134.5 (ArC), 131.9 (ArC), 128.0 (ArC), 124.6 (ArC), 123.8 (ArC), 115.2 (ArC), 71.0 (CH₂), 70.8 (CH₂), 70.8 (CH₂), 70.8 (CH₂), 70.1 (CH₂), 69.7 (CH₂), 67.9 (CH₂), 50.8 (CH₂); IR (thin film); 1704 (s), 2112 (m), 2864 (w) cm⁻¹; LRMS (ES⁺) 441.2 (100, [M+H]⁺); HRMS (ES⁺) calcd for C₂₂H₂₅N₄O₆ [M+H]⁺ 441.1747, observed 441.1762.

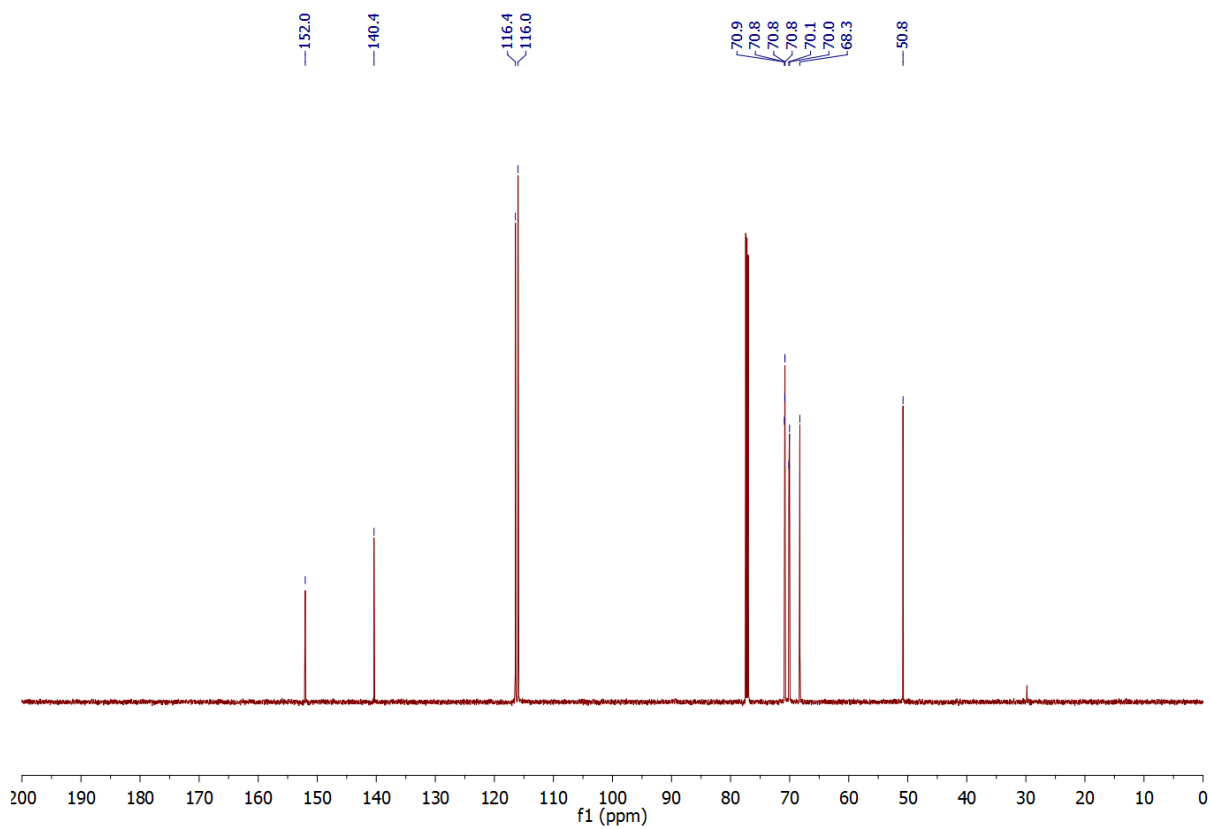
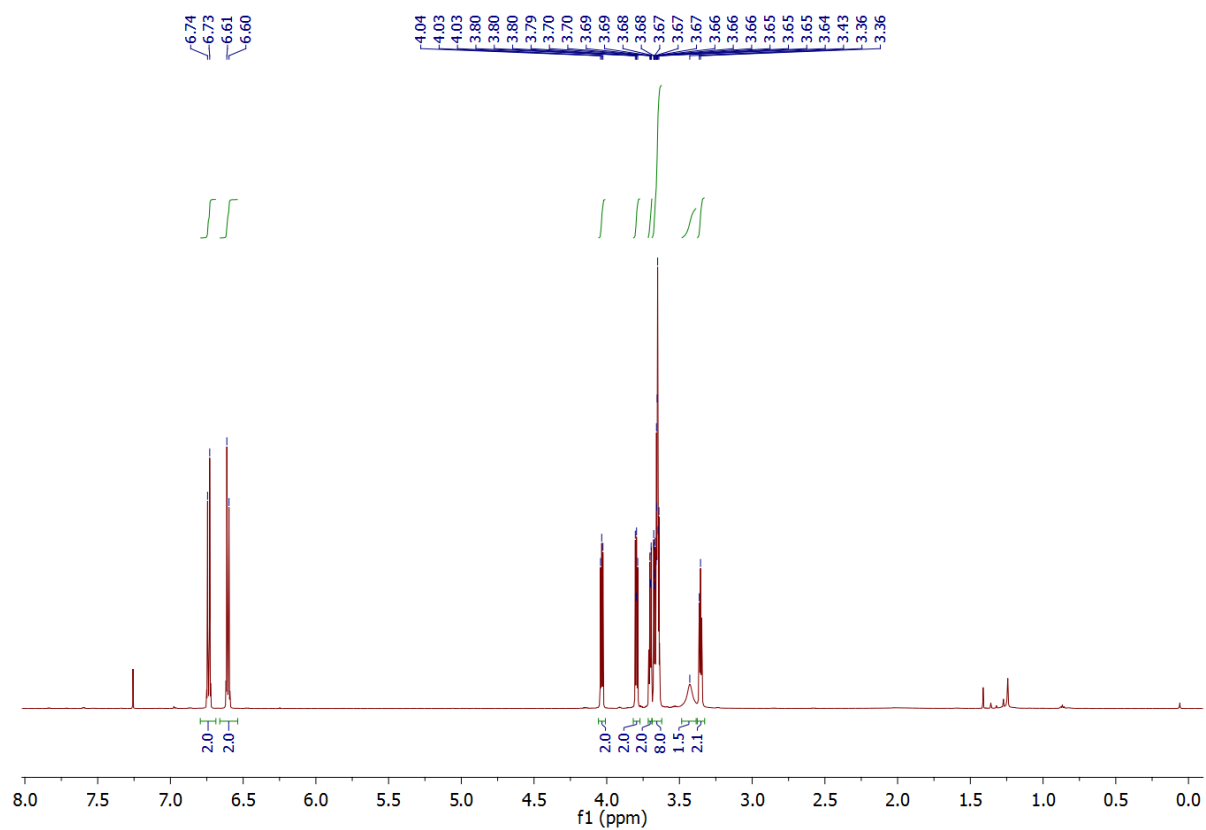


Synthesis of 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)aniline⁷ (**22**)

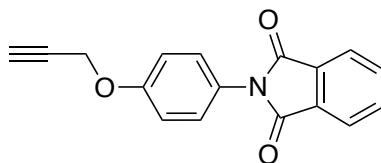


To phthalimide **33** (0.141 g, 0.320 mmol) in THF (10 mL) was added hydrazine monohydrate (330 μ L, 5.74 mmol, 60% solution). The reaction was stirred at room temperature for 2 h before water (30 mL) was added. The crude product was extracted with EtOAc (3 x 30 mL), washed with brine (20 mL), dried with MgSO_4 , filtered and the solvent removed on the rotary evaporator. The crude product was purified by column chromatography (20-100% EtOAc/petroleum ether) to yield the product as a brown oil (0.073 g, 0.233 mmol, 73%).

^1H NMR (600 MHz, CDCl_3); δ 6.74-6.73 (m, 2H, ArH), 6.61-6.60 (m, 2H, ArH), 4.04-4.03 (m, 2H, OCH_2), 3.80-3.79 (m, 2H, OCH_2), 3.70-3.69 (m, 2H, OCH_2), 3.68-3.64 (m, 8H, 4 x OCH_2), 3.43 (br s, 2H, NH_2), 3.36 (t, $J = 5.1$ Hz, 2H, CH_2N_3); ^{13}C NMR (151 MHz, CDCl_3); δ 152.0 (ArC), 140.4 (ArC), 116.4 (ArC), 116.0 (ArC), 70.9 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.1 (CH_2), 70.0 (CH_2), 68.3 (CH_2), 50.8 (CH_2); IR (thin film); 2098 (m), 2869 (m), 3356 (w), 3430 (w) cm^{-1} ; LRMS (ES^+) 311.2 (100, $[\text{M}+\text{H}]^+$); HRMS (ES^+) calcd for $\text{C}_{15}\text{H}_{19}\text{N}_8$ $[\text{M}+\text{H}]^+$ 311.1733, observed 311.1719.

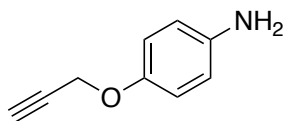


Synthesis of 2-(4-(prop-2-yn-1-yloxy)phenyl)isoindoline-1,3-dione⁸ (**34**)



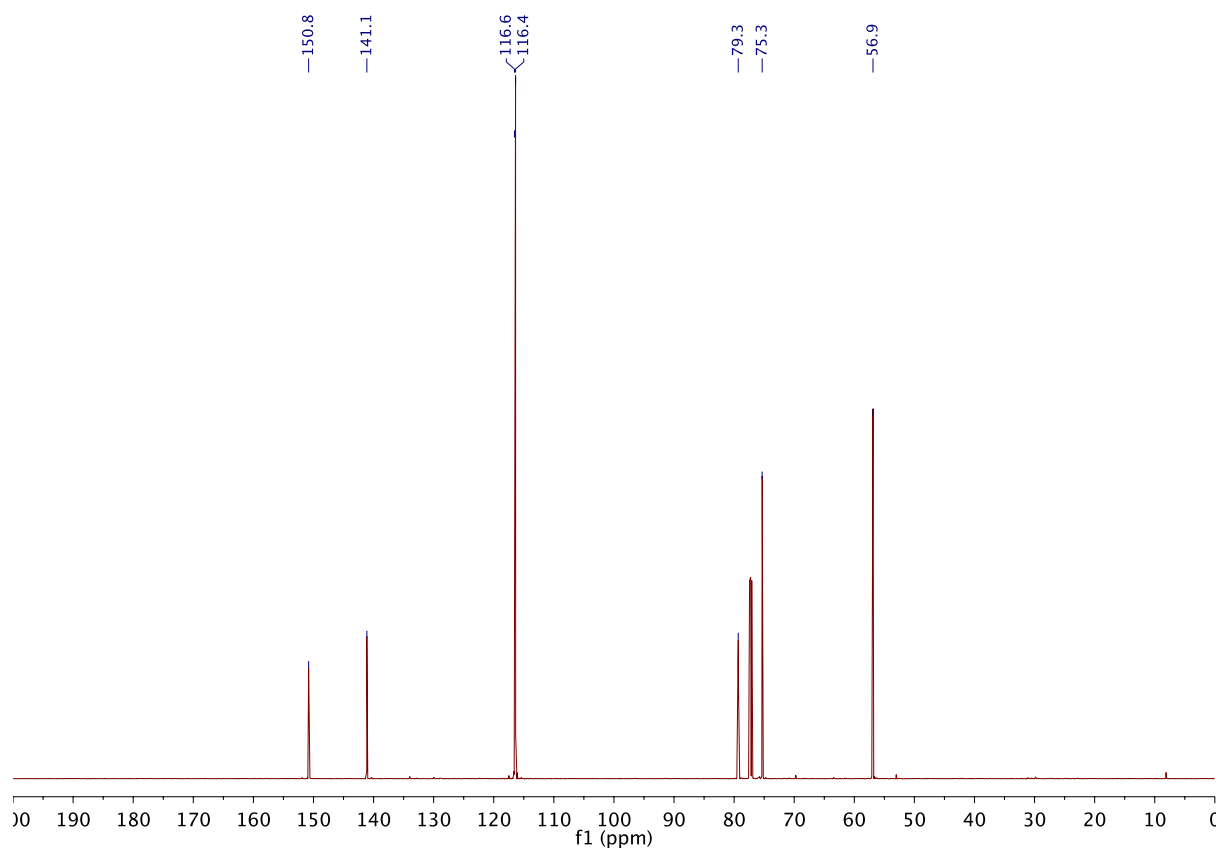
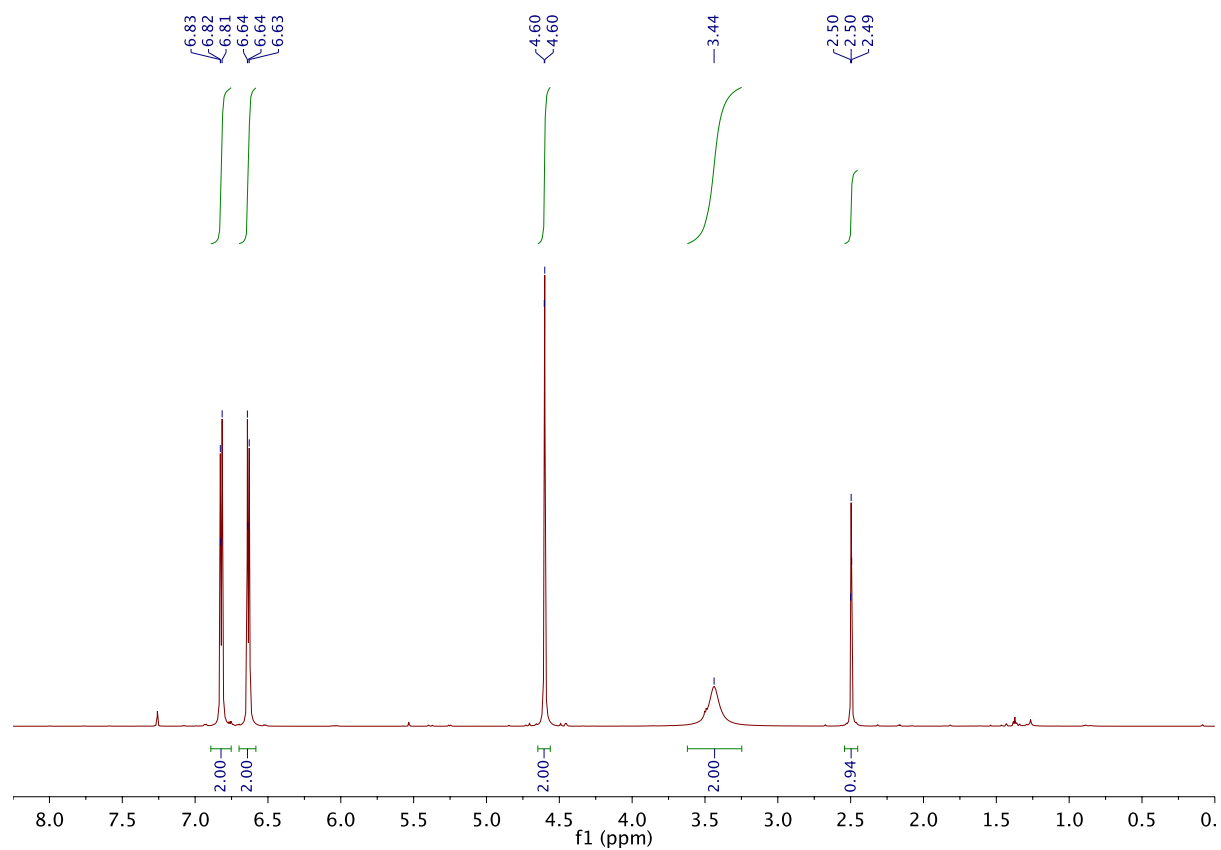
To phthalimide **30** (0.300 g, 1.254 mmol, 1.2 eq.) dissolved in 20 mL of DMF was added potassium tert-butoxide (1.15 mL, 1.149 mmol, 1.1 eq.). The mixture was stirred for 10 min before propargyl bromide (0.215 g, 1.045 mmol, 80% in toluene) was added to the reaction mixture and the resultant solution was refluxed and monitored by TLC. After 3 h, the reaction was quenched with water (100 mL). The organic phase was extracted with EtOAc (3 x 25 mL). Removal of solvent by evaporation afforded the crude product as a brown solid (0.386 g) which was used in the next step without further purification.

Synthesis of 4-(prop-2-yn-1-yloxy)aniline⁹ (**18**)



To the crude **34** (0.386 g) in THF (10 mL) was added hydrazine monohydrate (0.67 mL, 13.94 mmol, >10 eq.). The reaction mixture was stirred at rt and monitored by TLC. The reaction was quenched with water (30 mL) after 2 h. The organic phase was extracted with EtOAc (3 x 60 mL) and washed with brine (60 mL). The organic phase was dried over (MgSO₄), filtered and concentrated *in vacuo*. The crude was purified by column chromatography (20-50% EtOAc/petroleum ether) to afford the product as a dark oil (114.3 mg, 0.777 mmol, 74% over 2 steps).

¹H NMR (700 MHz, CDCl₃); δ 6.82 (d, *J* = 8.8 Hz, 2H, ArH), 6.64 (d, *J* = 8.8 Hz, 2H, ArH), 4.60 (d, *J* = 2.4, 2H, CH₂), 3.44 (s, 2H, NH₂), 2.49 (t, *J* = 2.4, 1H, HCC); ¹³C NMR (151MHz, CDCl₃); δ 150.8 (ArC), 141.1 (ArC), 116.6 (ArC), 116.4 (ArC), 79.3 (C), 75.3 (CH), 56.9 (CH₂); IR (thin film); 1203 (m); 1504 (m), 3280 (w), 3427-3353 (w) cm⁻¹; LRMS (ESI) 148.1 (100%, [M+H]⁺); HRMS (ES⁺) calcd for C₉H₉NO [M+H]⁺ 148.0684, observed 148.0757.



NMR Reactions

NMR reactions comparing amine reactivity with bromothiomaleimide 6:

Methyl S-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.002 g, 0.0047 mmol) was dissolved in CD₃CN (0.280 mL) in an NMR tube. Amine dissolved in phosphate buffer with 1% CD₃CN (2 eq., 0.700 mL, 13.5 mM amine, 100 mM phosphates, pH 7.4) was added to the organic solution (the resulting pH was then measured to be pH 7.8) and data acquisition carried out over the course of 24 h. Air inside a pasteur pipette was saturated with TMS vapour. The vapour was then bubbled through the NMR tube before the reaction was vortexed to ensure homogeneity of the reaction mixture. The reaction was left at room temperature and monitored by NMR. The ratio of the integrals of the starting material and product peaks could be calculated and then the progress of the reaction monitored until completion.

Table S3: Summary of NMR timecourse study.

R	Amine	Equiv.	Reaction Time (for 99%)	Efficiency
Me	Pyrrolidine	2	>900 min	Side products formed
Me	Aniline	2	786 min	Quantitative conversion
Me	<i>p</i> -Anisidine	2	185 min	Quantitative conversion
Me	<i>N</i> -Me <i>p</i> -anisidine	2	186 min	Quantitative conversion

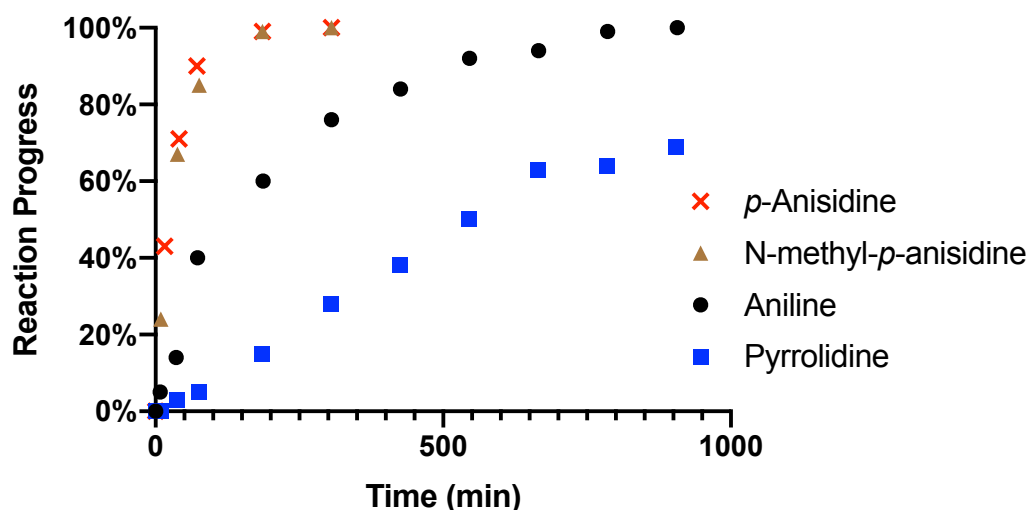


Figure S1: Timecourse NMR study carried out in phosphate buffer solution at pH 7.8. Peak integration used to measure reaction progress.

NMR reactions comparing *p*-anisidine with analogues **18** and **22** in reaction with bromothiomaleimide **6**.

Amine (2 eq.) was dissolved in CD₃CN (250 μL) and phosphate buffer (700 μL, 100 mM, pH 6.75) was added to the methyl 3-((4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)thio)-2-((tert-butoxycarbonyl)amino)propanoate (0.0025 g, 0.0059 mmol, 1 eq.) dissolved in CD₃CN (250 μL) in a NMR tube. The pH of the final solution was measured at 7.4. The reaction was monitored by NMR. The ratio of the integrals of the starting material and product peaks could be calculated and then the progress of the reaction monitored until completion.

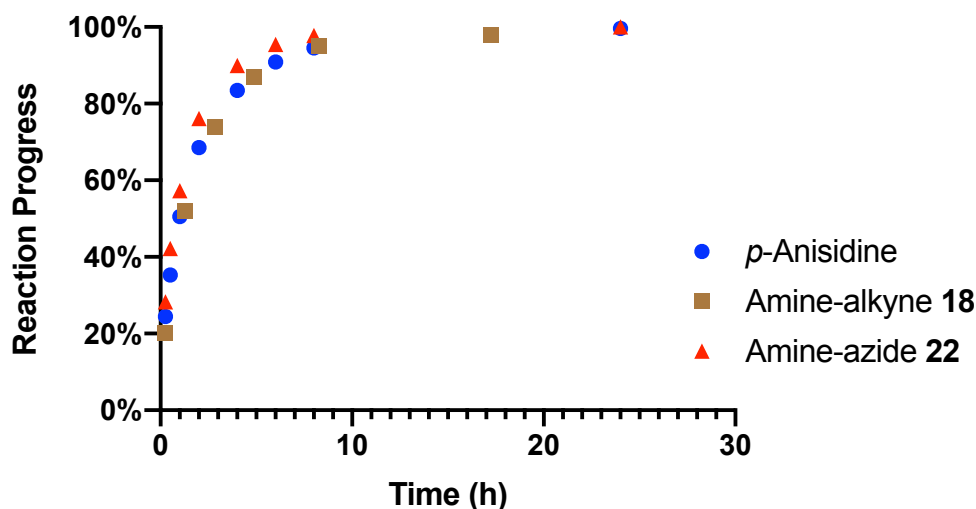


Figure S2: Timecourse NMR study carried out in phosphate buffer solution at pH 7.4. Peak integration used to measure reaction progress.

Protein and Peptide Mass Spectra

Protein samples:

HSA samples were buffer swapped into H₂O before being diluted to 4 µM for MS analysis. For MS analysis, capillary liquid chromatography mass spectrometry (CapLC-MS) was performed on an Agilent 1100/1200 LC system connected to a 6510A QTOF mass spectrometer (Agilent, UK). 10 µL of sample (4 µM) was injected onto an Agilent PLRP-S (150 mm x 2.1 mm, 1000 Å, 8 µm) column. Two LC-MS methods were used with the same LC column and mobile phases A (H₂O with 0.1% formic acid) and B (MeCN, 0.1% formic acid).

Method A:

The LC column was heated to 60 °C. The gradient elution was as follows: 25% B (isocratic for 1 min) increase to 99% B in 16 min and stay at 99% B for 2 min following a sharp decrease to 25% B in 0.1 min and isocratic for 1.9 min at 25% B. The flow rate was 0.25 mL/min. Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 4000V, a gas temperature at 325 °C, a dry gas flow rate at 5 L/min and a nebulizer of 20 psig. MS ToF was acquired under conditions of a fragmentor at 175 V, a skimmer at 65 V, QuadAMU at 140, an octopole RF peak at 750 and an acquisition rate of 1 spectra/s in profile mode, within a scan range between 100 and 3100 m/z. The .d data was then analysed by deconvoluting the spectrum to a zero charge mass spectra using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

Method B:

The LC column was heated to 60 °C. The gradient elution was as follows: 25% B for 1 min followed by increase to 99% B over 16 min. After 2 min, 99% B was decreased to 25% over 0.1 min and maintained at 25% B for 1.9 min. The flow rate was 0.3 mL/min. Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 4000V, a gas temperature at 325 °C, a dry gas flow rate at 5 L/min and a nebulizer of 20 psig. MS ToF was acquired under conditions of a fragmentor at 175 V,

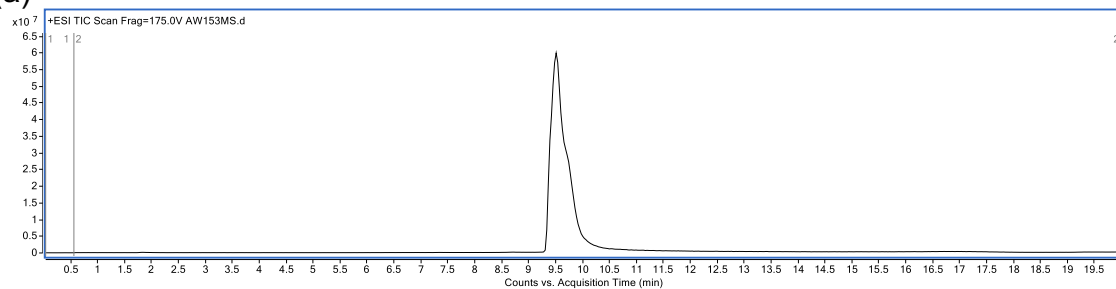
a skimmer at 65 V, QuadAMU at 140, an octopole RF peak at 750 and an acquisition rate of 1 spectra/s in profile mode, within a scan range between 100 and 3100 m/z. The .d data was then analysed by deconvoluting the spectrum to a zero charge mass spectra using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

Peptide Samples:

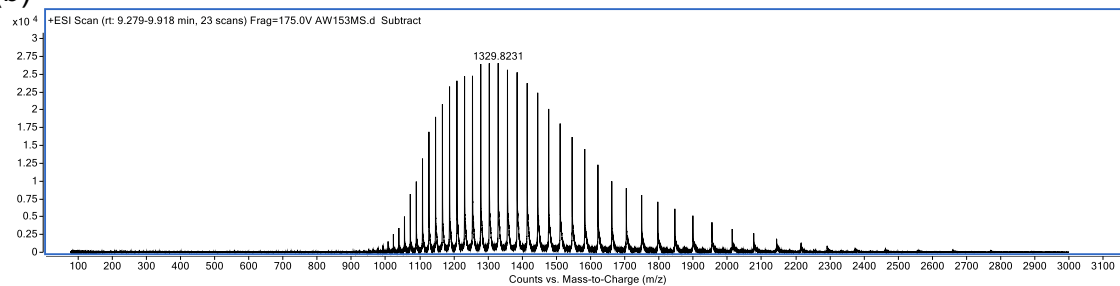
LCMS was performed on samples using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD). All samples were run with the following parameters. Column: Hypersil Gold C4, 1.9 μm , 2.1 μm \times 50 μm . Wavelength: 254 nm. Mobile Phase: 95:5 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid) Gradient over 4 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.6 mL/min. MS Mode: ES+. Scan Range: m/z = 250 – 2000. Scan time: 0.25 s. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the appropriate range. Total mass spectra for protein samples were reconstructed from the ion series using the MaxEnt 1 algorithm pre-installed on MassLynx software.

Native WT HSA

(a)



(b)



(c)

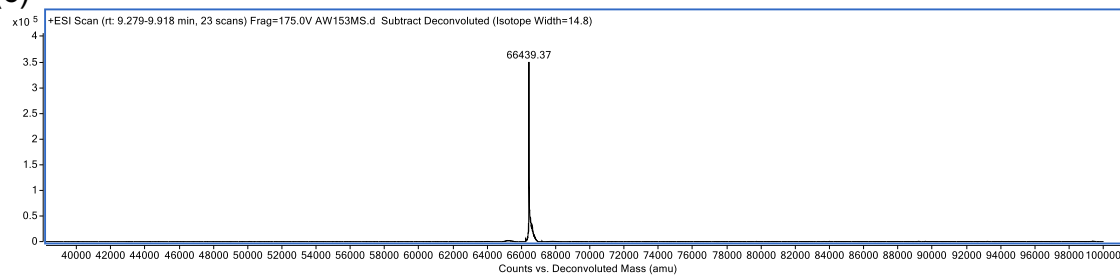
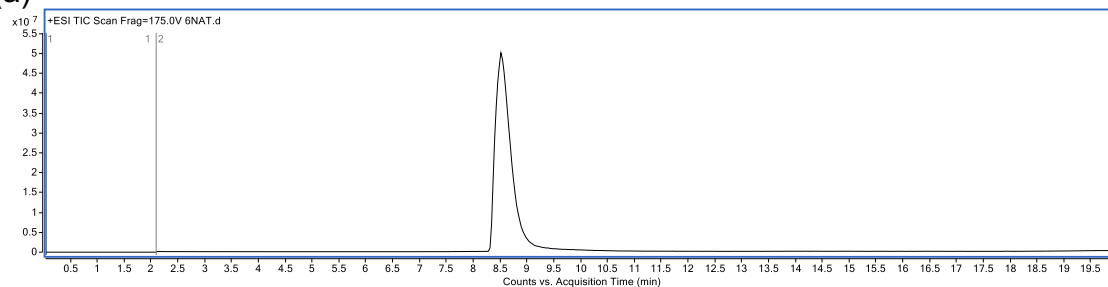


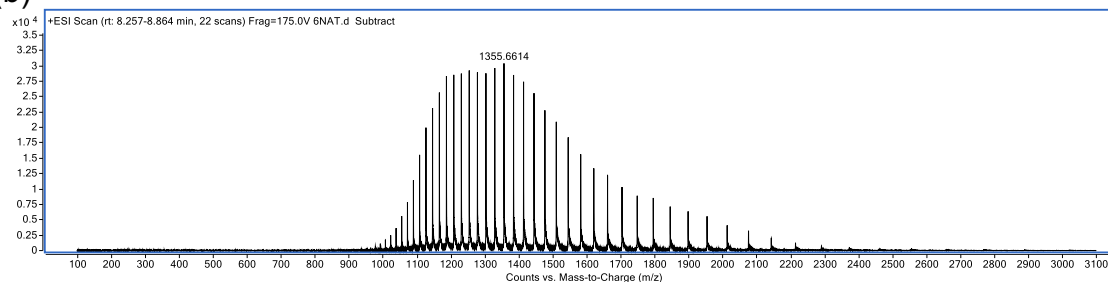
Figure S3: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native WT HSA. Expected mass: 66439 Da. Observed mass: 66439 Da. Data obtained with MS method A.

Native mHSA

(a)



(b)



(c)

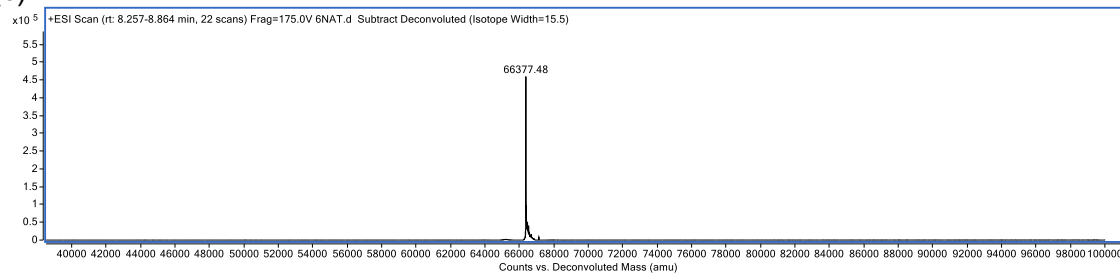


Figure S4: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for mHSA. Expected mass: 66381 Da. Observed mass: 66377 Da. Data obtained with MS method B.

Expression and Reduction of GFP: Cysteine Mutant Green Fluorescent Protein (GFPS147C)

GFPS147C was expressed in competent *E. coli* BL21(DE3) cells as described previously.¹⁰ Due to the presence of a small amount of dimer species, GFPS147C was then reduced prior to conjugation and characterised as below:

TCEP (31.3 μ L, 20 mM in deionised water, 25 equiv.) was added to a solution of GFPS147C (500 μ L, 50 μ M) in PBS (pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 90 min. Excess reagents were removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units) into PBS (pH 7.4, 5 mM EDTA) for further experiments. Samples were desalted (7 kDa MWCO, ZebaSpin®) prior to LCMS analysis. Concentration was determined photometrically using $\epsilon_{280} = 20,500 \text{ M}^{-1} \text{ cm}^{-1}$.

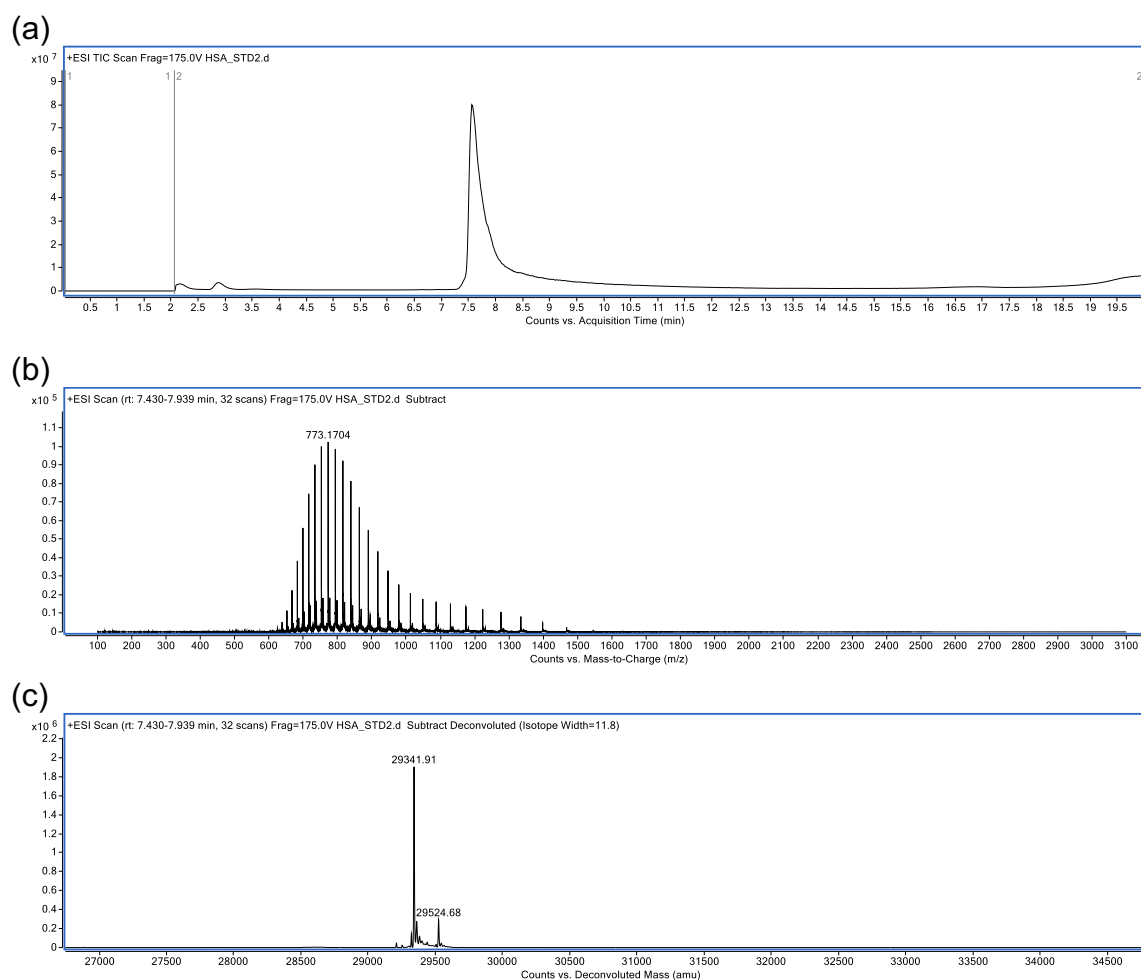
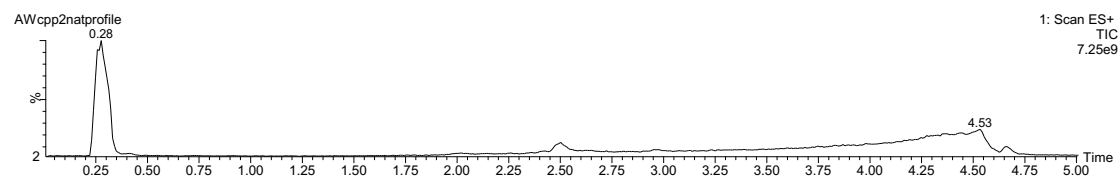


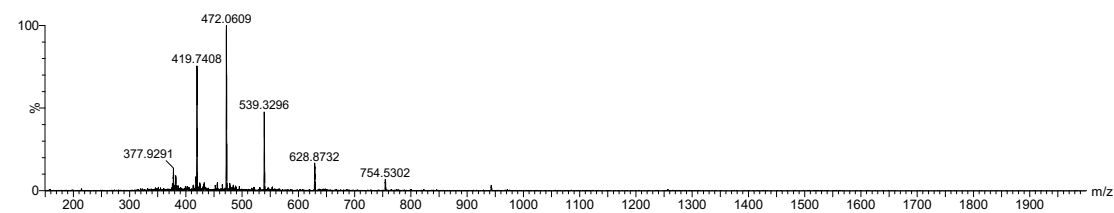
Figure S5: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for reduced GFP. Observed mass: 29342 Da and 29525 Da. Data obtained with MS method B.

Native CPP

(a)



(b)



(c)

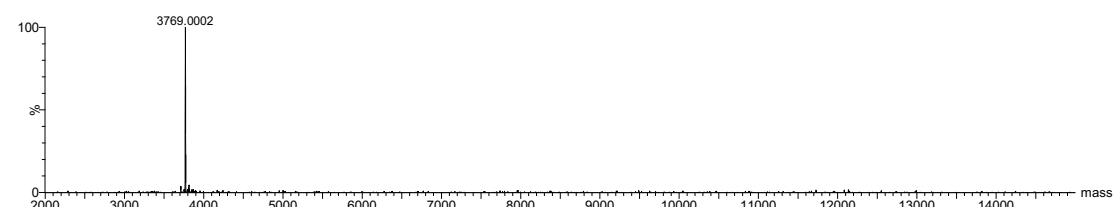
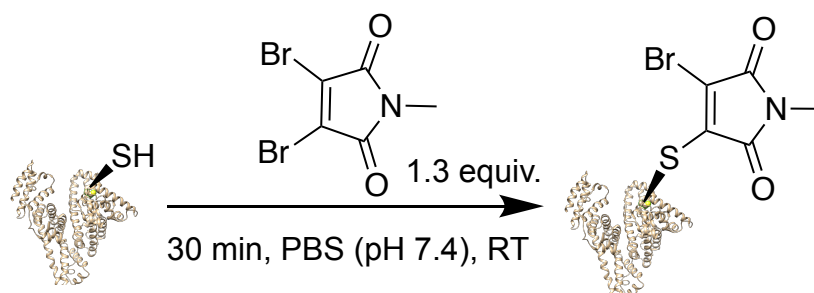


Figure S6: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native CPP in H₂O. Expected mass: 3765 Da. Observed mass: 3769 Da. Data obtained with peptide LCMS method.

WT HSA, *N*-Me DBM (**14**)



N-Me DBM **13** (1.3 μ L, 10 mM, 1.3 equiv. in DMSO) was added to HSA (100 μ M, 100 μ L) in PBS buffer (12 mM phosphates, pH 7.4) at RT. After 30 minutes, excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

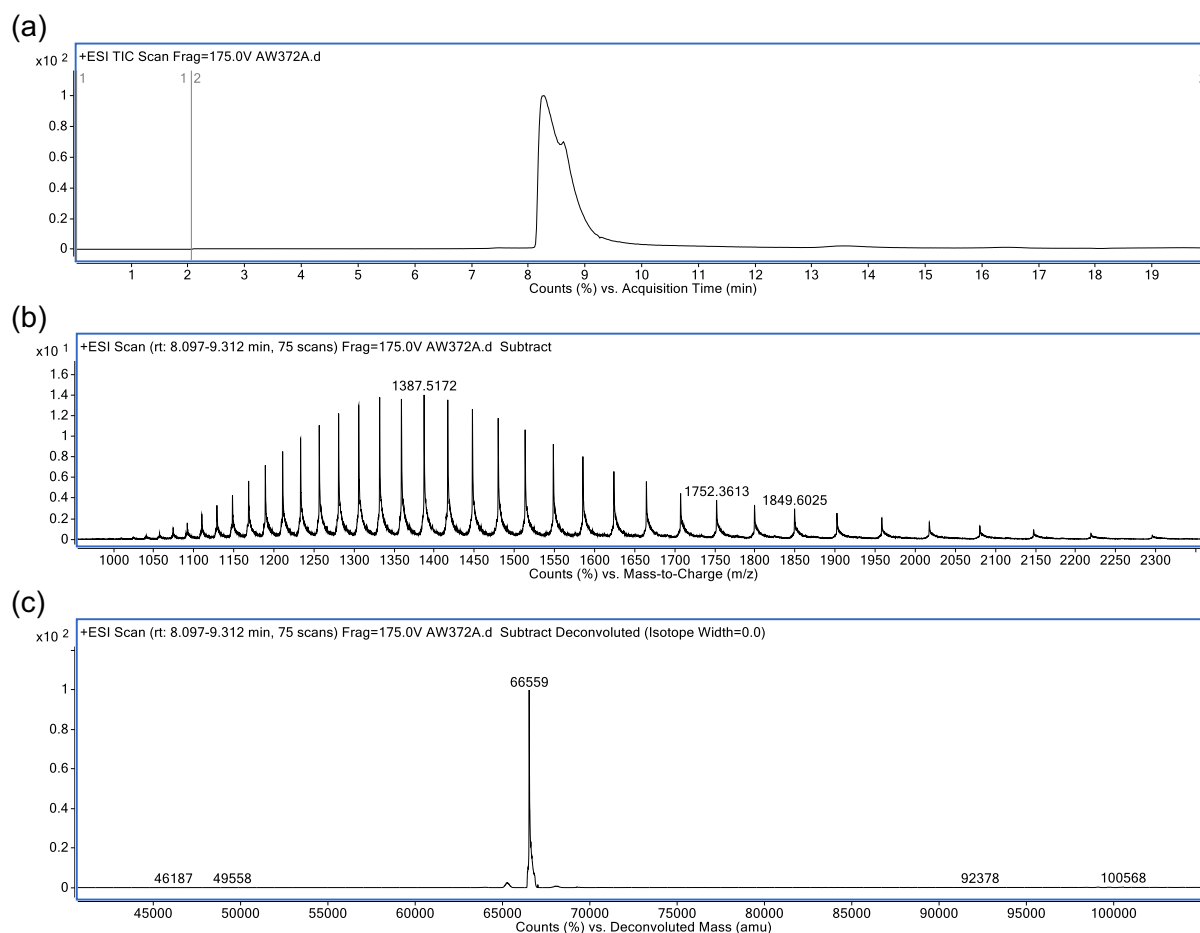
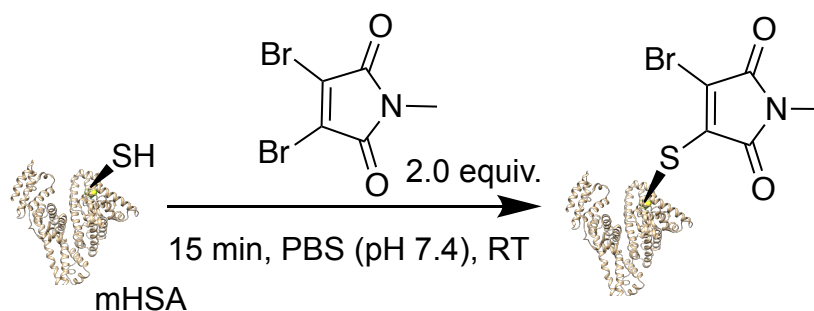


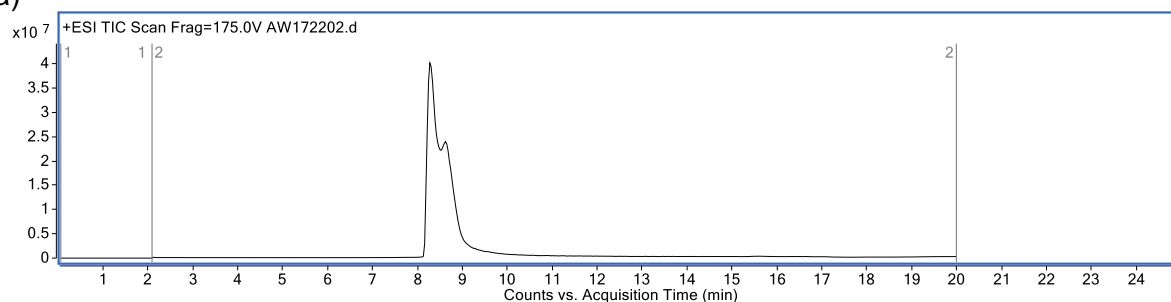
Figure S7: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for *N*-Me DBM **13** conjugated to WT HSA. Expected mass: 66548 Da (loss of Br). Observed mass: 66559 Da (loss of Br + partial hydrolysis). Data obtained with MS method B.

mHSA, N-Me DBM (15)

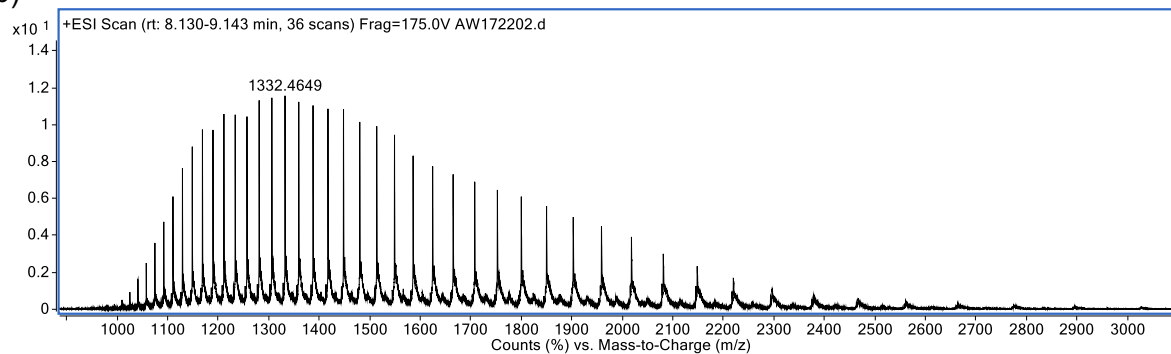


N-Me DBM **13** (3.3 μ L, 10 mM, 2.0 equiv. in DMSO) was added to HSA (165 μ M, 100 μ L) in PBS buffer (12 mM phosphates, pH 7.4) at RT. After 15 minutes, excess small molecule was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were then analysed by LCMS.

(a)



(b)



(c)

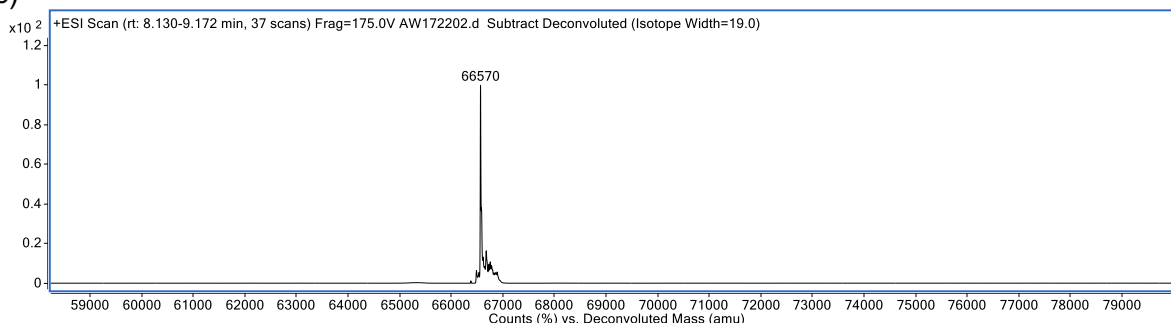
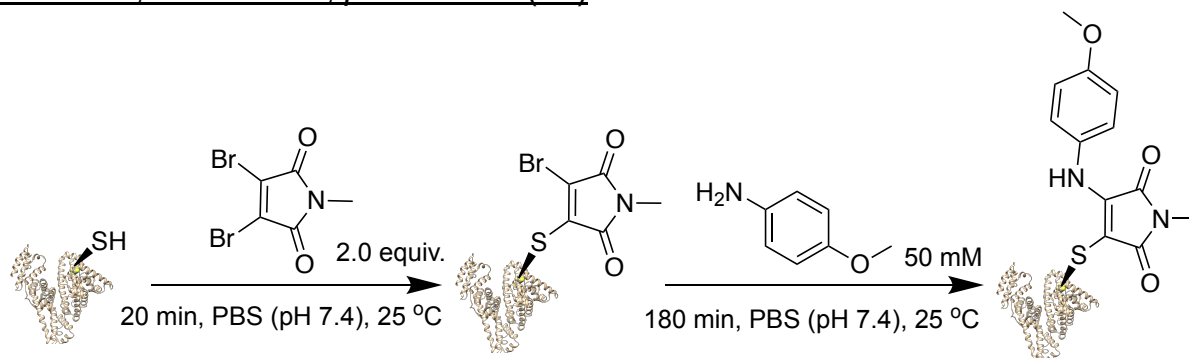


Figure S8: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to mHSA. Expected mass: 66570 Da. Observed mass: 66570 Da. Data obtained with MS method B.

WT HSA, *N*-Me DBM, *p*-Anisidine (**16**)



N-Me DBM **13** (3.6 μ L, 10 mM, 2.0 equiv. in DMSO) was added to WT HSA (100 μ M, 180 μ L) in PBS buffer (12 mM phosphates, pH 7.4) in a quartz cuvette and monitored by spectrophotometer at 25 °C. After the increase in absorbance at 375 nm had stopped (20 minutes), *p*-anisidine (2.9 μ L, 3.2 M in DMSO) was added, and the reaction was returned to the spectrophotometer at 25 °C. The absorbance increase at 415 nm had stopped after a further 180 minutes (UV analysis was continued until minute 946), excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

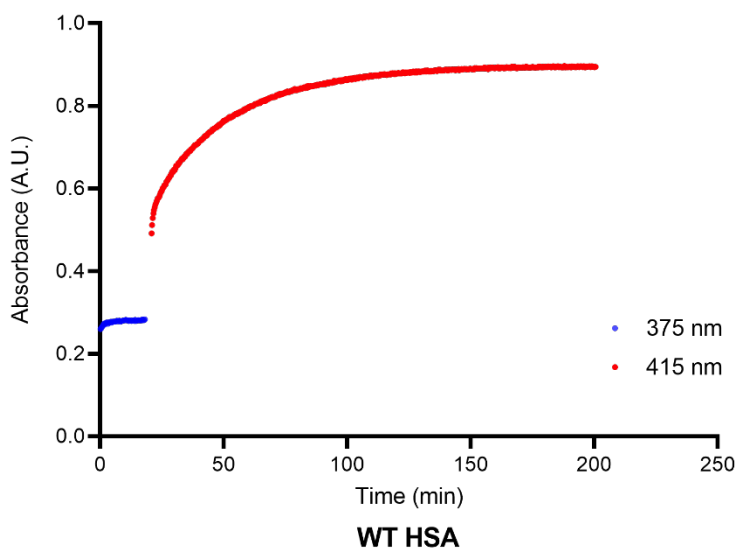


Figure S9: UV trace of DBM **13** conjugation to WT HSA, followed by addition of *p*-anisidine.

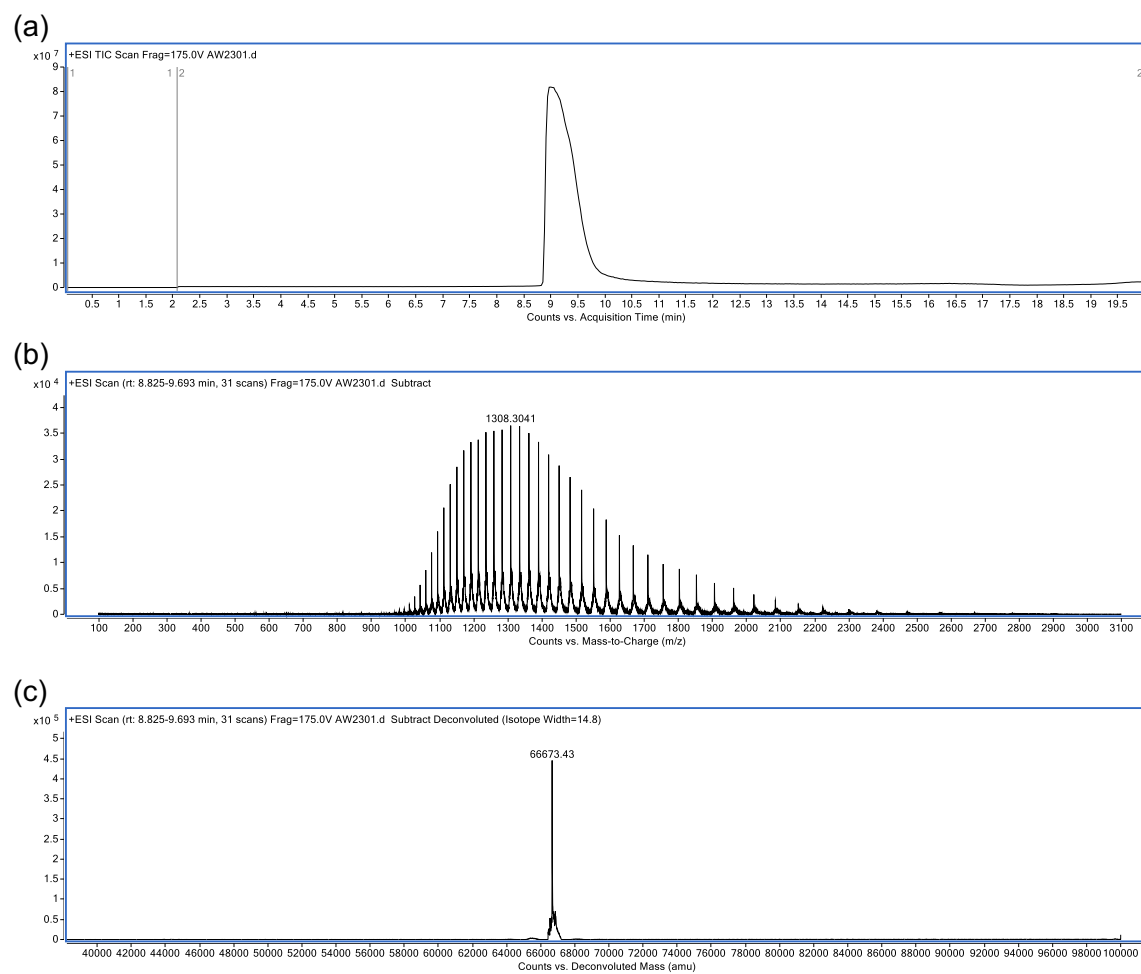
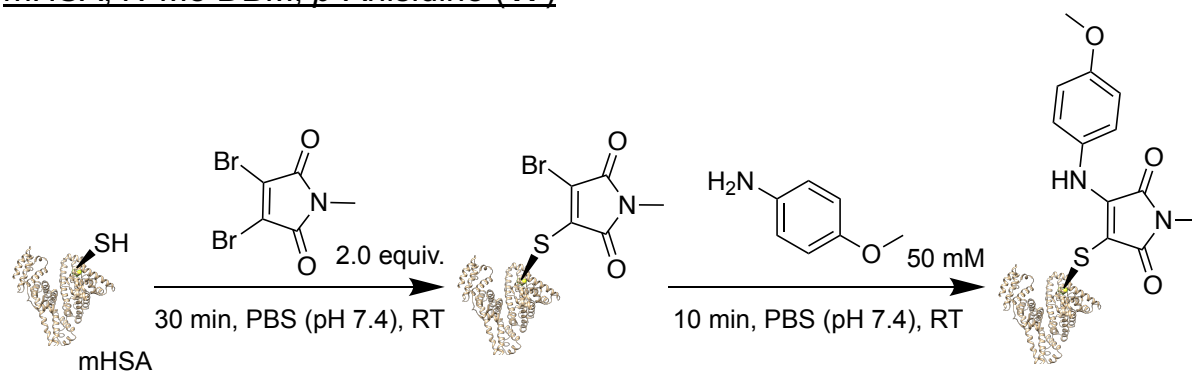


Figure S10: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for *N*-Me DBM **13** conjugated to WT HSA with subsequent addition of *p*-anisidine. Expected mass: 66671 Da. Observed mass: 66673 Da. Data obtained with MS method B.

mHSA, *N*-Me DBM, *p*-Anisidine (17)



N-Me DBM **13** (3.6 μ L, 10 mM, 2.0 equiv. in DMSO) was added to mHSA (100 μ M, 180 μ L) in PBS buffer (12 mM phosphates, pH 7.4) in a quartz cuvette and monitored by spectrophotometer at ambient temperature (23 $^{\circ}$ C). After the increase in absorbance at 375 nm had stopped (30 minutes), *p*-anisidine (2.9 μ L, 3.2 M in DMSO) was added, and the reaction was returned to the spectrophotometer at 23 $^{\circ}$ C. The absorbance increase at 415 nm had stopped after a further 10 minutes (UV analysis was continued until minute 45), excess small molecule was removed (7 kDa MWCO, ZebaSpin[®]). Conjugates were then analysed by LCMS.

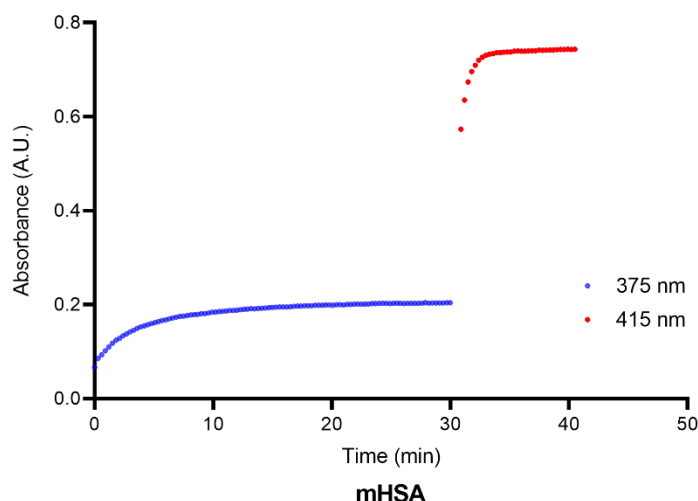


Figure S11: UV trace of DBM **13** conjugation to mHSA, followed by addition of *p*-anisidine.

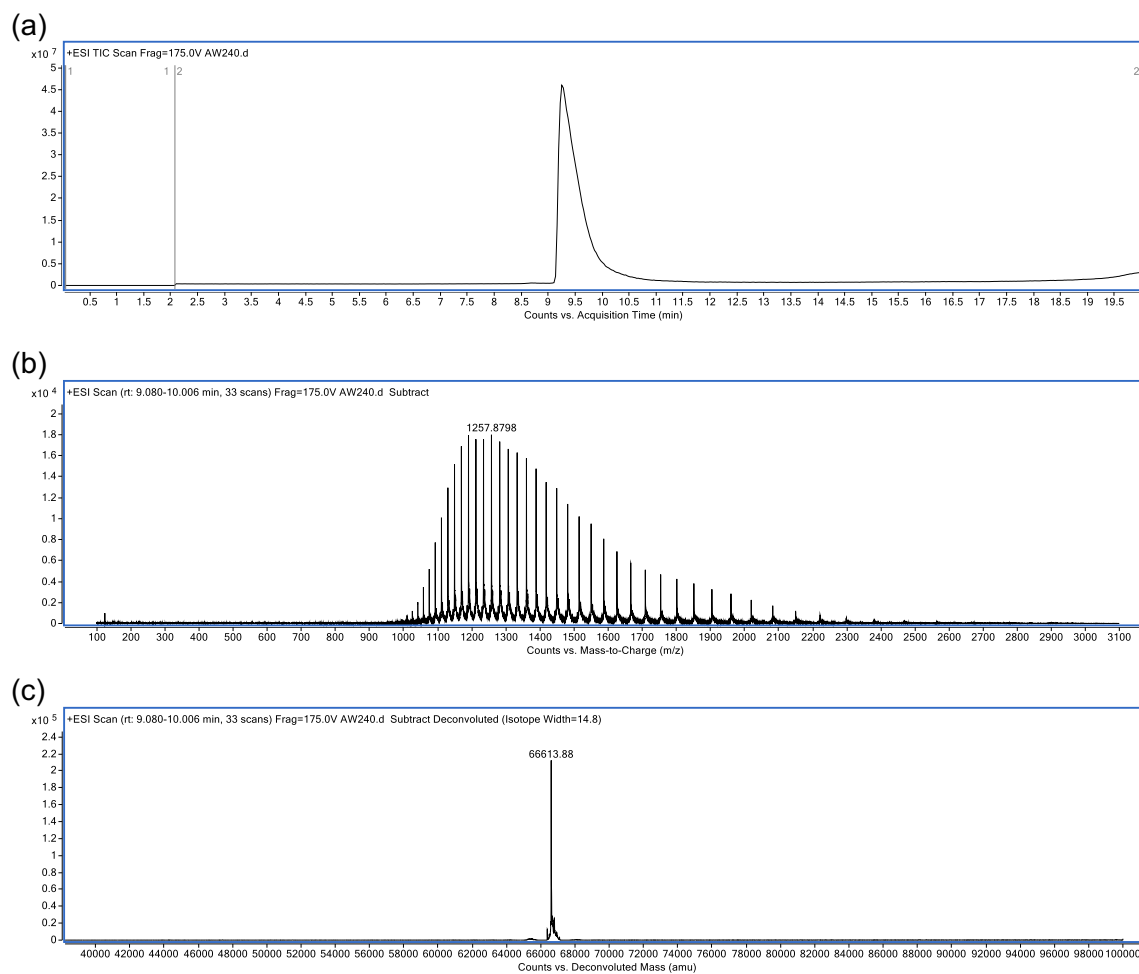
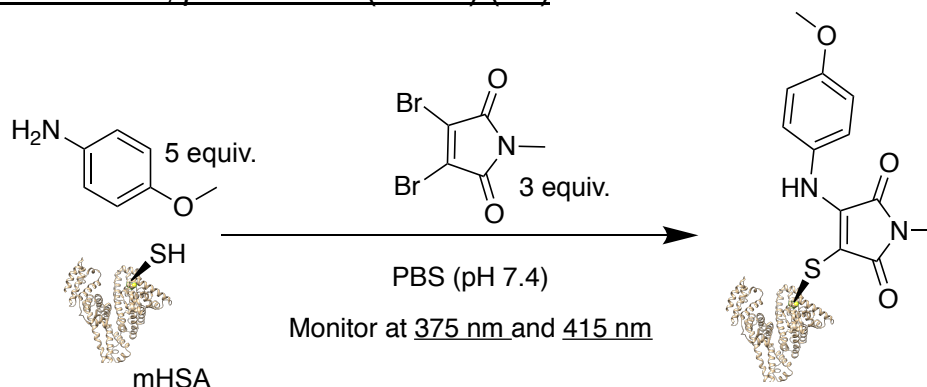


Figure S12: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to mHSA with subsequent addition of *p*-anisidine. Expected mass: 66612 Da. Observed mass: 66614 Da. Data obtained with MS method B.

mHSA, *N*-Me DBM, *p*-Anisidine (*in situ*) (17)



p-Anisidine (2.81 μ L, 32 mM, 5.0 equiv. in DMSO) was added to mHSA (100 μ M, 180 μ L) in PBS buffer (12 mM phosphates, pH 7.4). *N*-Me DBM **13** (5.4 μ L, 10 mM, 3.0 equiv. in DMSO) was added and the reaction left at room temperature overnight. Excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were analysed by LCMS.

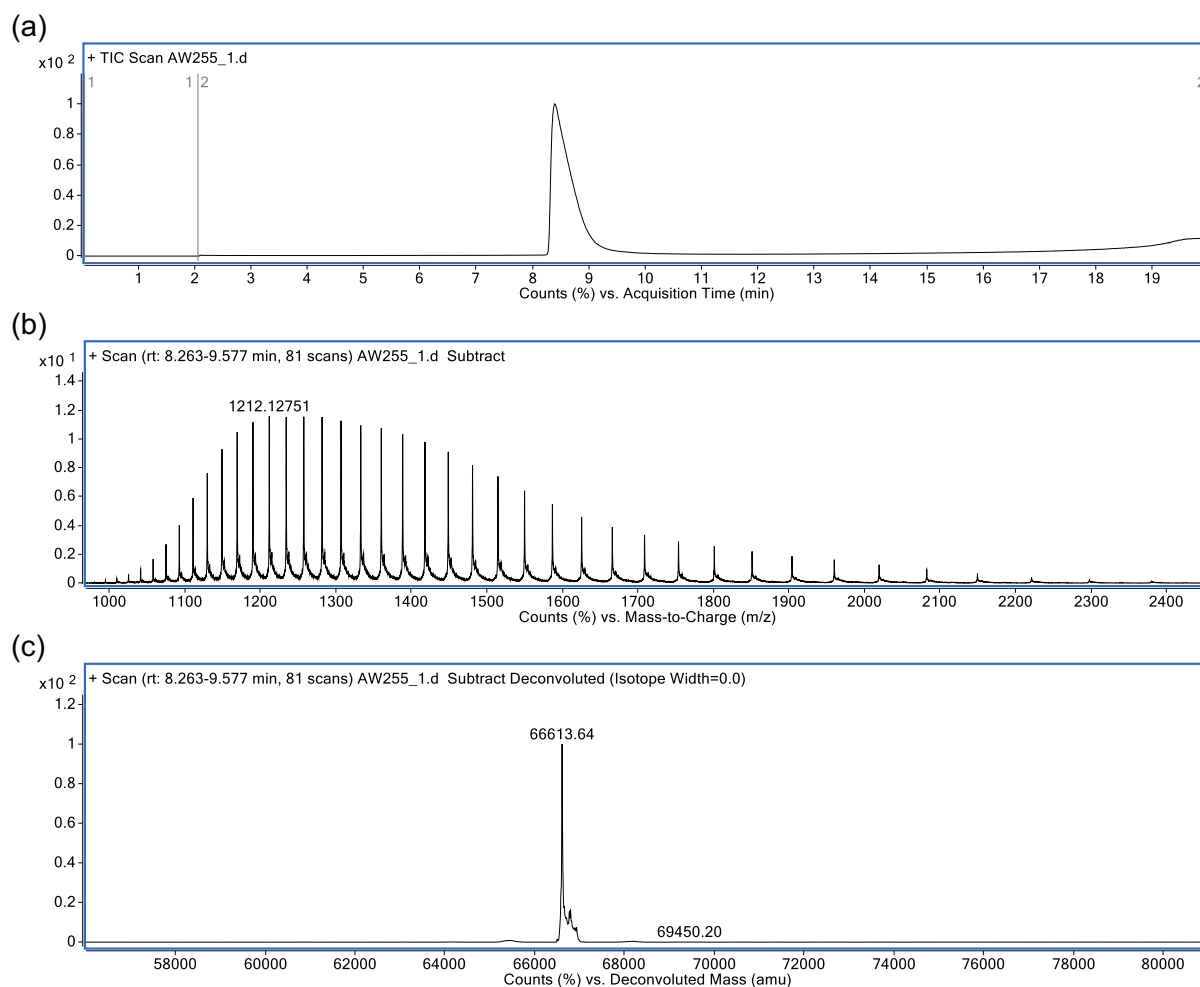
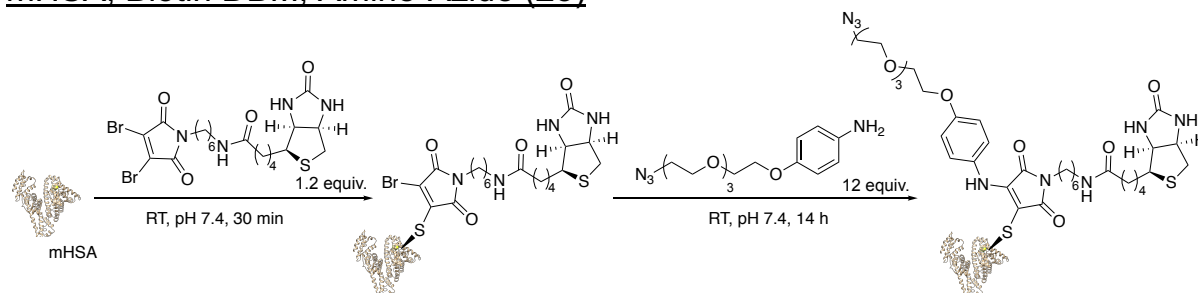


Figure S13: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for *in situ* *N*-Me DBM **13** conjugated to mHSA with *p*-anisidine. Expected mass: 66612 Da. Observed mass: 66614 Da. Data obtained with MS method B.

mHSA, Biotin DBM, Amine-Azide (**23**)



DBM **21** (0.9 μ L, 10 mM, 1.2 equiv. in DMSO) was added to mHSA (100 μ M, 75 μ L) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 30 minutes, amine-azide **22** (0.28 μ L, 0.32 M, 12.0 equiv. in DMSO) was added, and the reaction was left at RT for 14 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

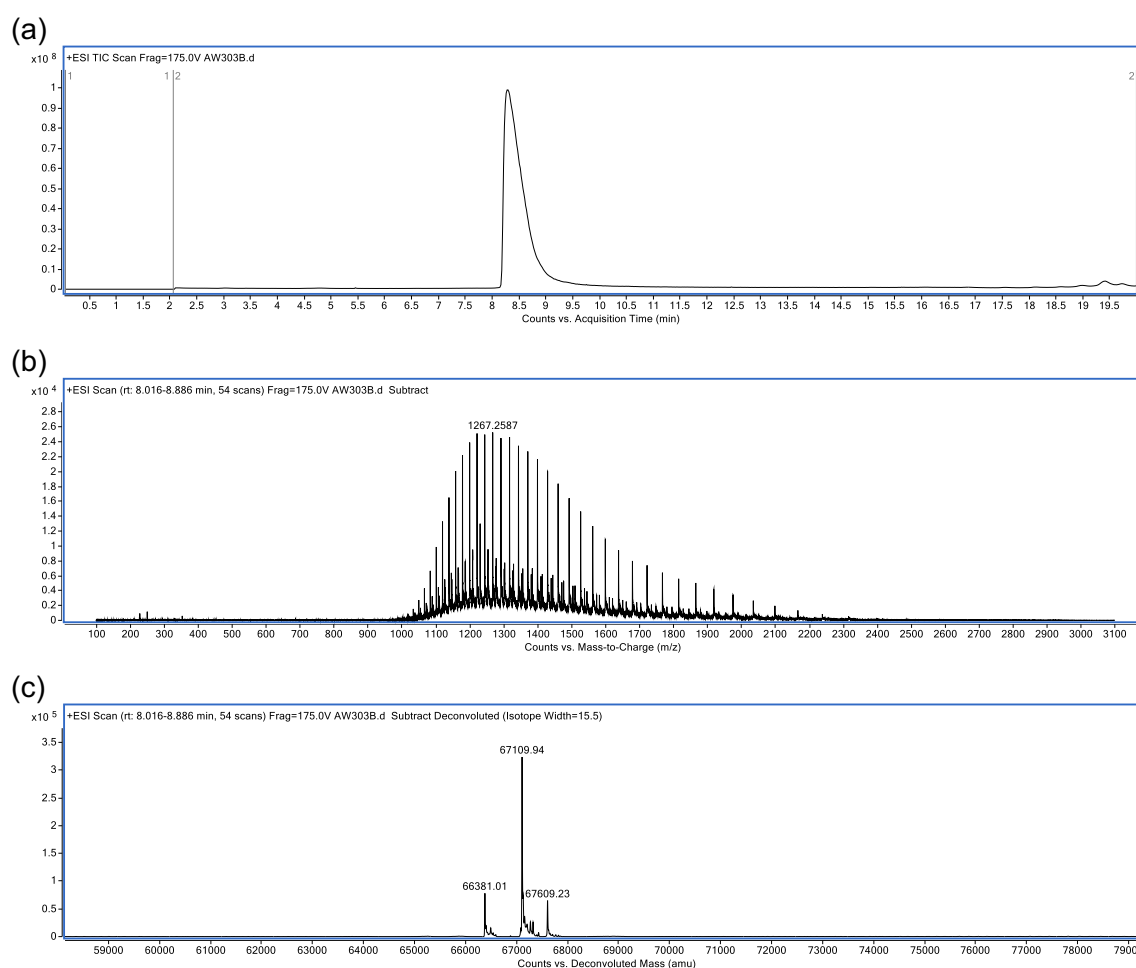
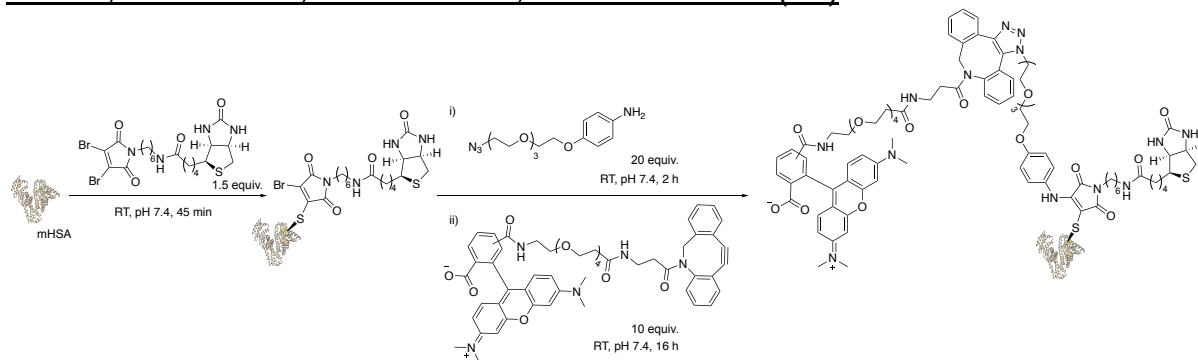


Figure S14: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to mHSA with subsequent addition of amine-azide **22**. Expected mass: 67111 Da. Observed mass: 67110 Da. Native protein expected mass: 66381 Da. Observed mass: 66381 Da. Non-specific modification expected mass: 67611 Da. Observed mass: 67609 Da. Data obtained with MS method B.

mHSA, Biotin DBM, Amine-Azide, TAMRA-DBCO (24)



DBM **21** (1.5 μ L, 10 mM, 1.5 equiv. in DMSO) was added to mHSA (100 μ M, 100 μ L) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 45 minutes, amine-azide **22** (0.20 μ L, 1.0 M, 20 equiv. in DMSO) was added, and the reaction was left at RT for 2 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). TAMRA-DBCO (2.0 μ L, 10 mM, 10 equiv. in DMF) was added to 20 μ L of the resultant solution, and left at room temperature for 16 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

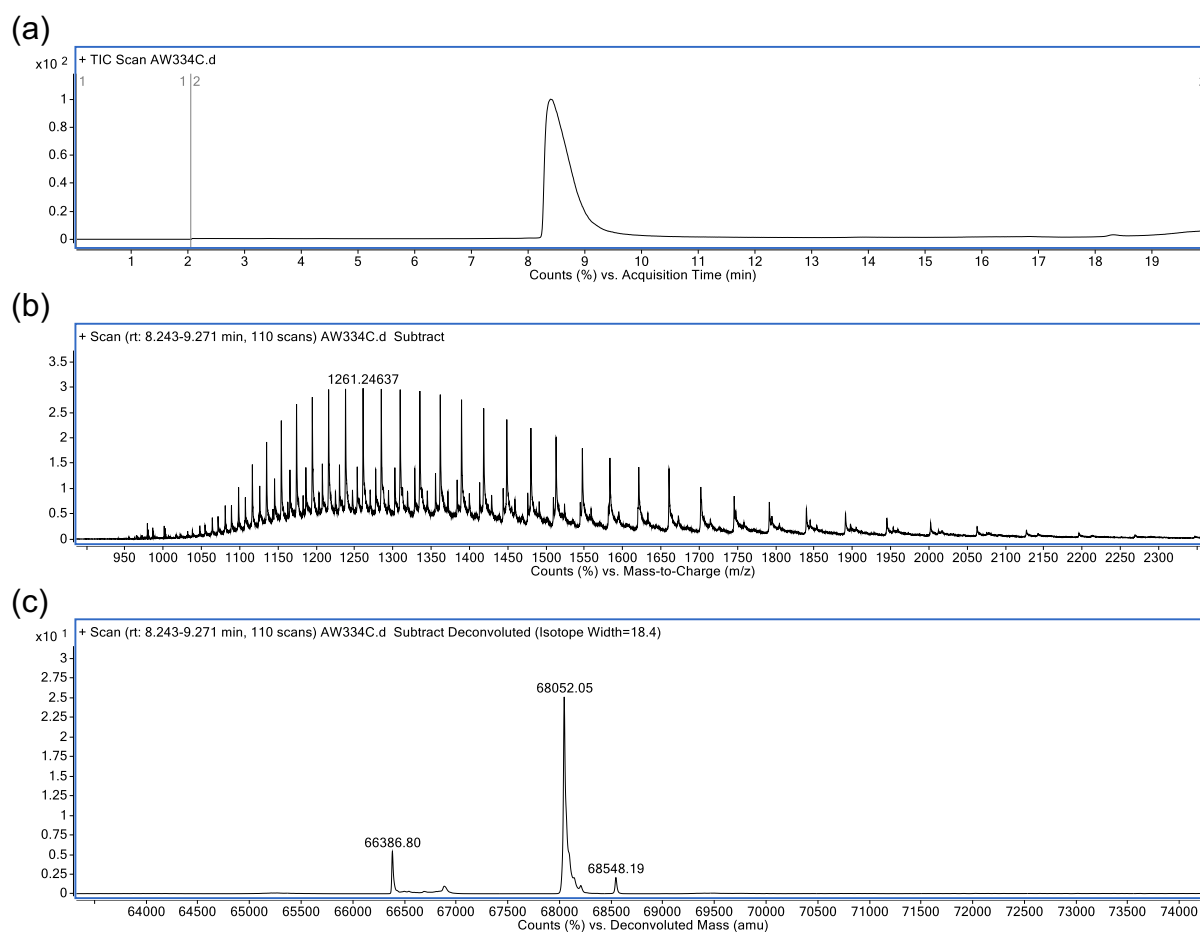
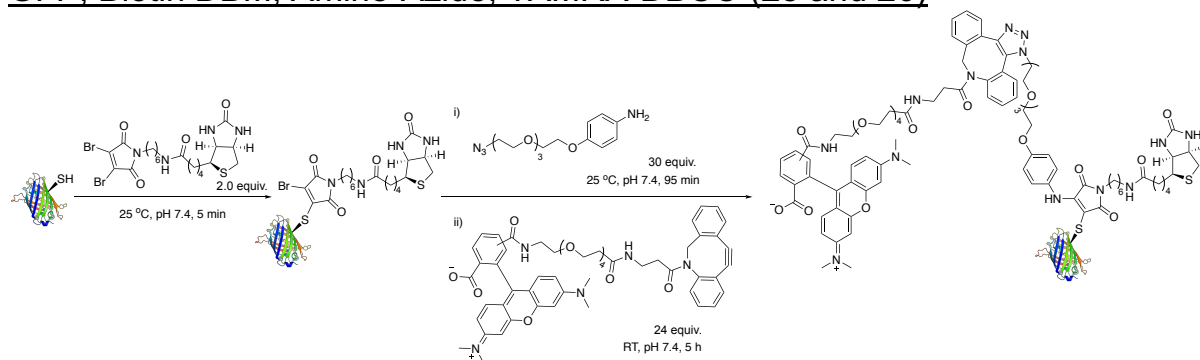


Figure S15: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to mHSA with subsequent addition of amine-azide **22** and TAMRA-DBCO. Expected mass: 68047 Da. Observed mass: 68052 Da. Native protein expected mass: 66381 Da. Observed mass: 66387 Da. Non-specific modification expected mass: 68547 Da. Observed mass: 68548 Da. Data obtained with MS method B.

GFP, Biotin DBM, Amine-Azide, TAMRA-DBCO (**25** and **26**)



DBM **21** (1.44 μL , 10 mM, 2.0 equiv. in DMSO) was added to reduced GFP (40 μM , 180 μL) in PBS buffer (12 mM phosphates, pH 7.4, 5 mM EDTA) in a quartz cuvette and monitored by spectrophotometer at 25 °C. After the increase in absorbance at 375 nm had stopped (5 minutes), amine-azide **22** (2.16 μL , 0.1 M, 30 equiv. in DMSO) was added, and the reaction was returned to the spectrophotometer at 25 °C. When the absorbance increase at 415 nm had stopped (a further 95 minutes), excess small molecule was removed (7 kDa MWCO, ZebaSpin®), and the intermediate amino-thio conjugate was analysed by LCMS. The resultant solution was then diluted to 32 μM with PBS buffer (12 mM phosphates, pH 7.4). TAMRA-DBCO (10.0 μL , 10 mM, 24 equiv. in DMF) was added to 130 μL of the resultant solution, and left at room temperature for 5 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

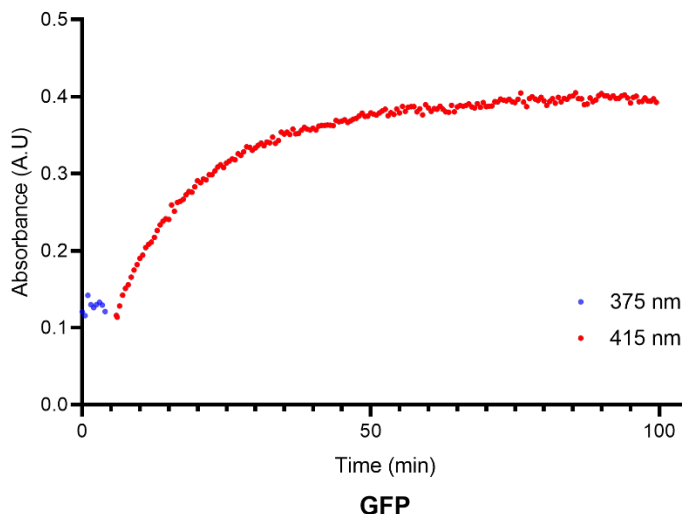


Figure S16: UV trace of DBM **21** conjugation to GFP, followed by addition of amine azide **22**.

Intermediate amino-thio conjugate (**25**):

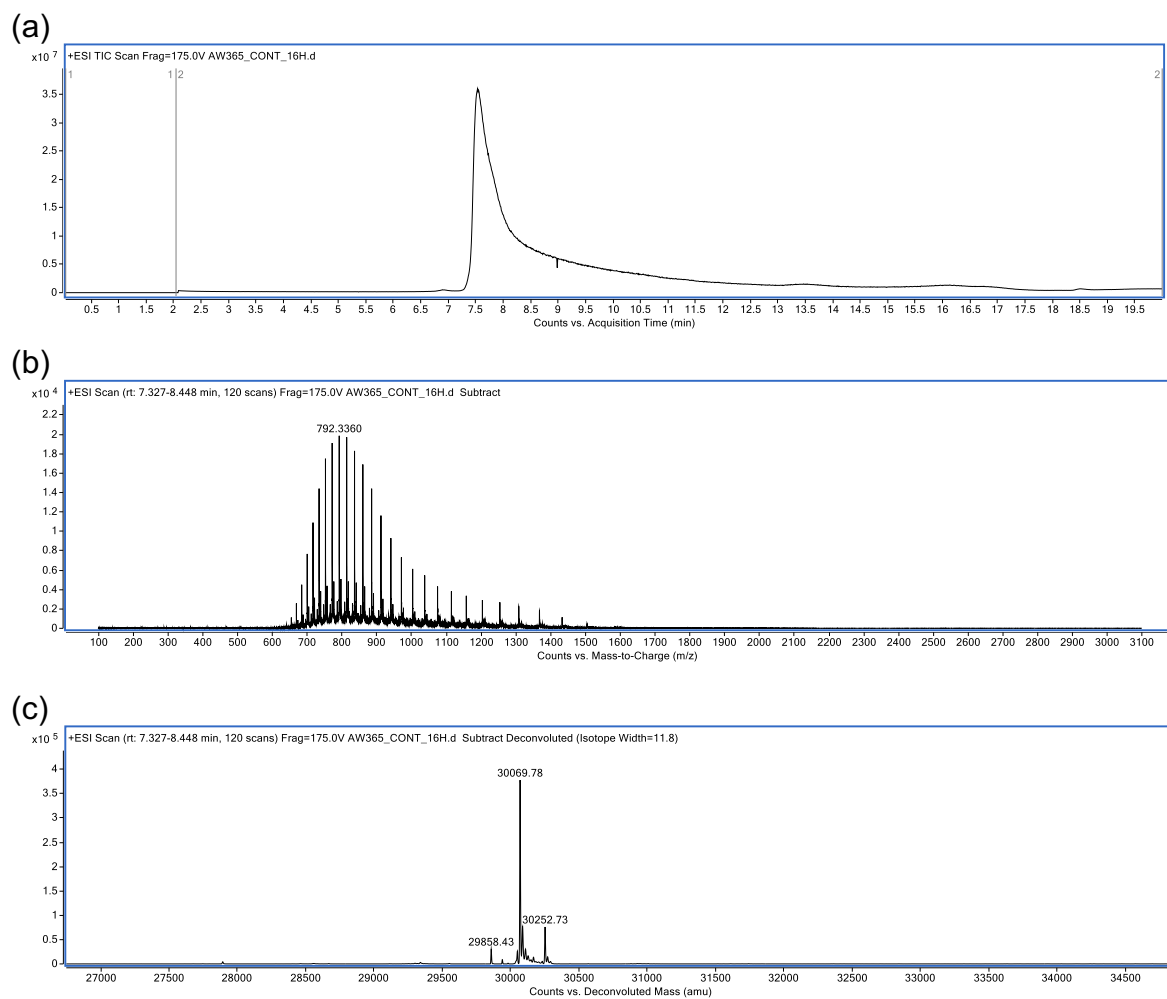


Figure S17: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to GFP with subsequent addition of amine-azide **22**. Expected mass: 30072 Da and 30254 Da. Observed mass: 30070 Da and 30253 Da. Data obtained with MS method B.

Final conjugate (**26**):

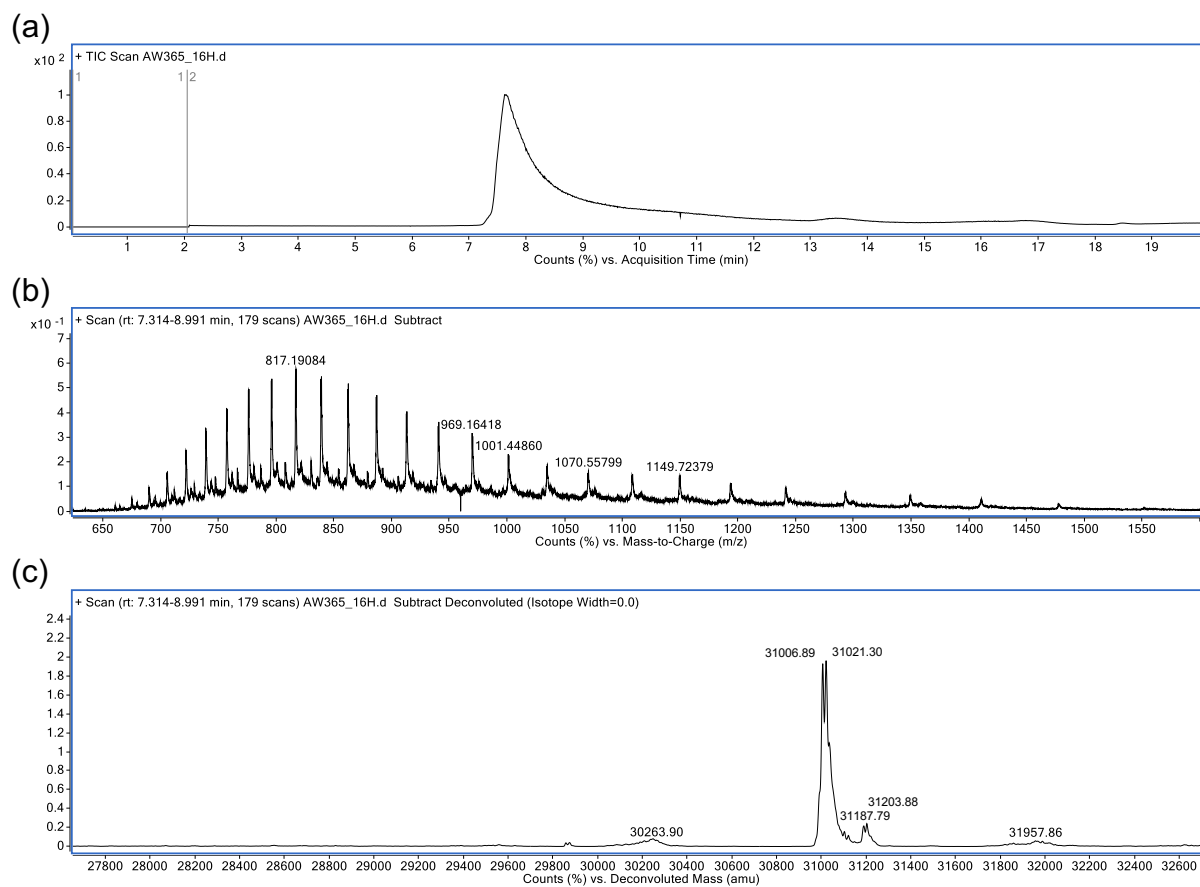
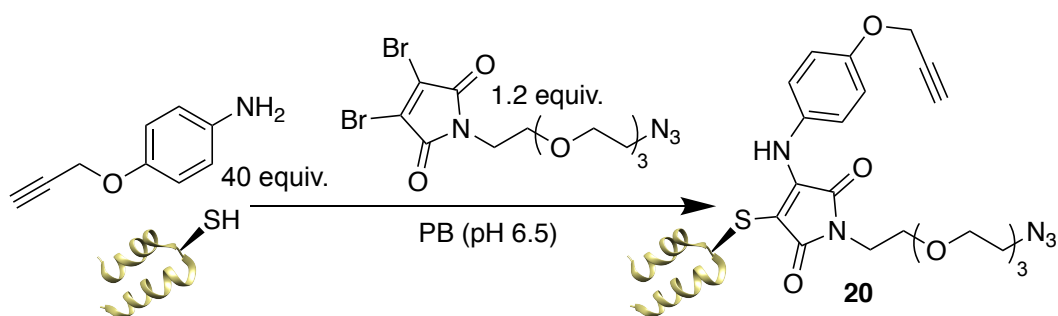


Figure S18: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to GFP with subsequent addition of amine-azide **22** and TAMRA-DBCO. Expected mass: 31008 Da and 31190 Da. Observed mass: 31007 Da and 31188 Da. Oxidation observed at 31021 and 31204 Da, this is known to occur with methionine-rich proteins¹¹ and crucially is not observed in the amino-thio intermediate. Data obtained with MS method B.

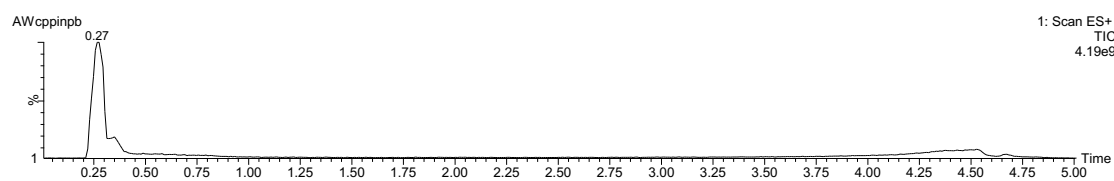
CPP, Azide DBM, Amine-Alkyne (*in situ*) (20)



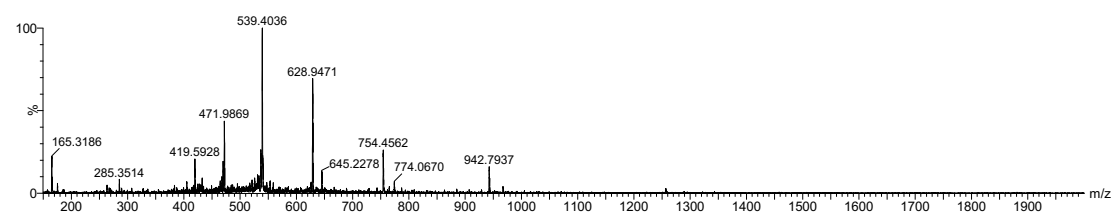
Amine-alkyne **18** (10 μ L, 100 mM, 40 equiv. in DMSO) was added to CPP (0.9 mM, 25 μ L) in PB buffer (10 mM phosphates, pH 6.5). DBM **19** (3 μ L, 10 mM, 1.2 equiv. in DMSO) was added and the reaction left at 37 $^{\circ}$ C for 30 min. The crude reaction mixture was analysed by LCMS. Purification by HPLC was carried out before conjugates were analysed again by LCMS.

Native peptide (in 10 mM PB):

(a)



(b) 0.20-0.40 min of LC trace:



(c)

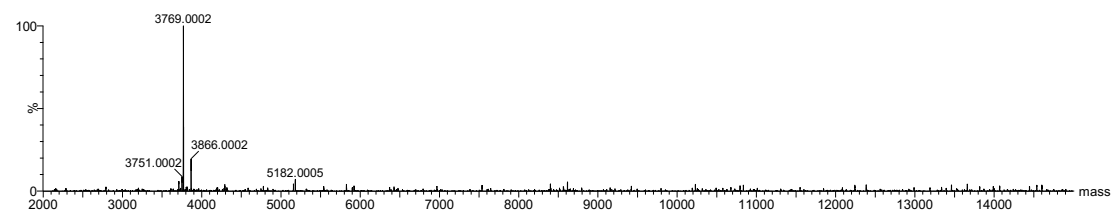
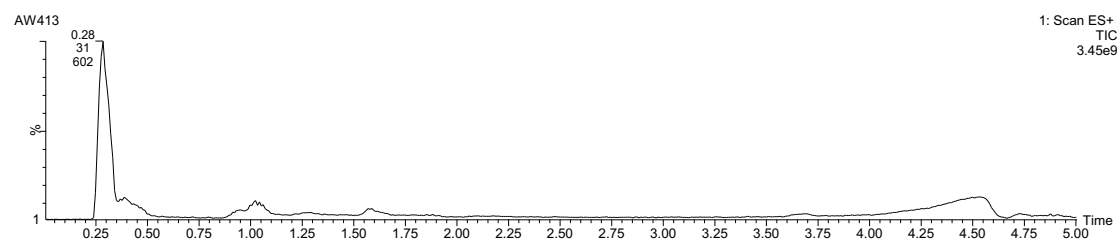


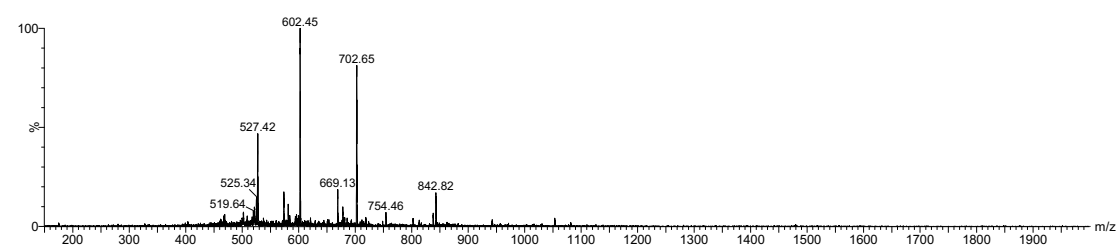
Figure S19: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native CPP in PB. Expected mass: 3765 Da. Observed mass: 3769 Da. Data obtained with peptide LCMS method.

LC of reaction mixture (in 10 mM PB):

(a)



(b) 0.25-0.35 min of LC trace:



(c)

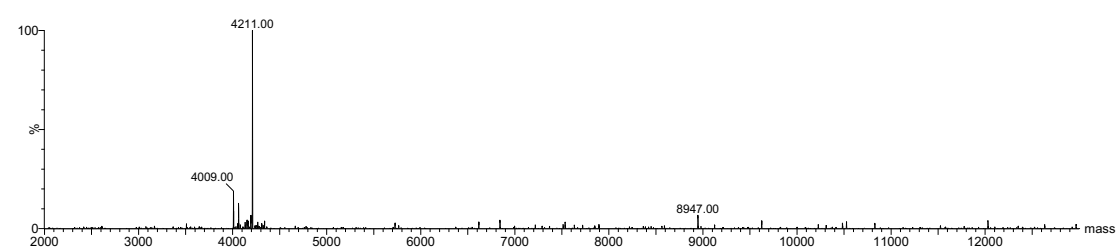
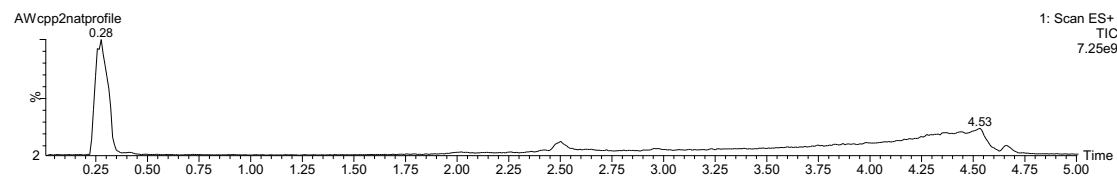


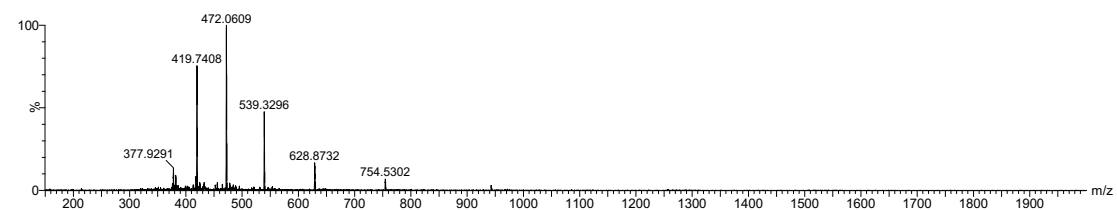
Figure S20: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for crude *in situ* DBM **19** conjugated to CPP with amine alkyne **18**. Expected mass: 4208 Da. Observed mass: 4211 Da. Data obtained with peptide LCMS method.

Native peptide (in H₂O):

(a)



(b) 0.25-0.30 min of LC trace:



(c)

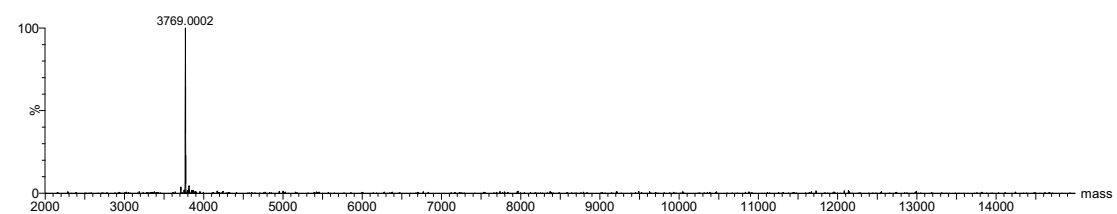
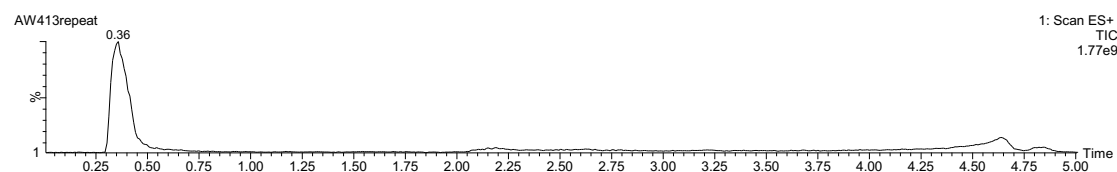


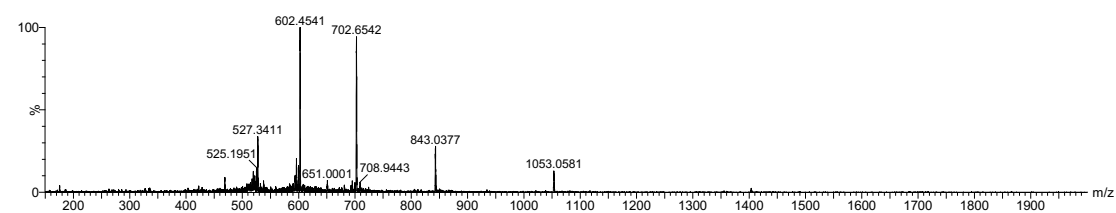
Figure S21: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native CPP in H₂O. Expected mass: 3765 Da. Observed mass: 3769 Da. Data obtained with peptide LCMS method.

Post HPLC (in H₂O) (**20**):

(a)



(b) 0.30-0.50 min of LC trace:



(c)

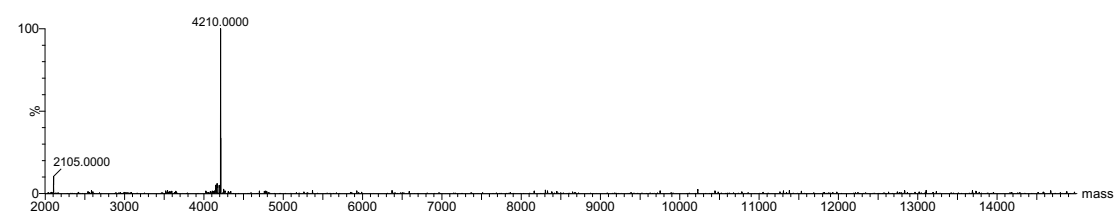
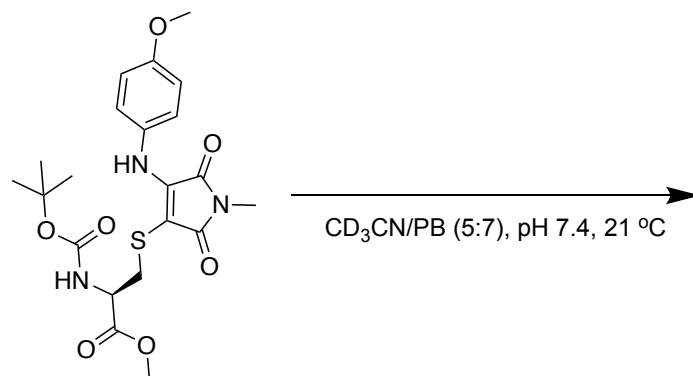


Figure S22: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **20**. Expected mass: 4208 Da. Observed mass: 4210 Da. Data obtained with peptide LCMS method.

Stability Experiments

Small Molecule Study

Buffer stability (pH 7.4)



Aminothiomaieimide **11** (4.30 μmol , 500 μL , 8.60 mM in CD_3CN) was added to PB (700 μL , 100 mM phosphates). The pH was corrected to 7.4 and the solution was incubated at 21 °C for 6 days. The solution was analysed by NMR. The *N*-Me peak at 2.98 ppm was monitored carefully to observe if a reaction occurred. No reaction was observed.

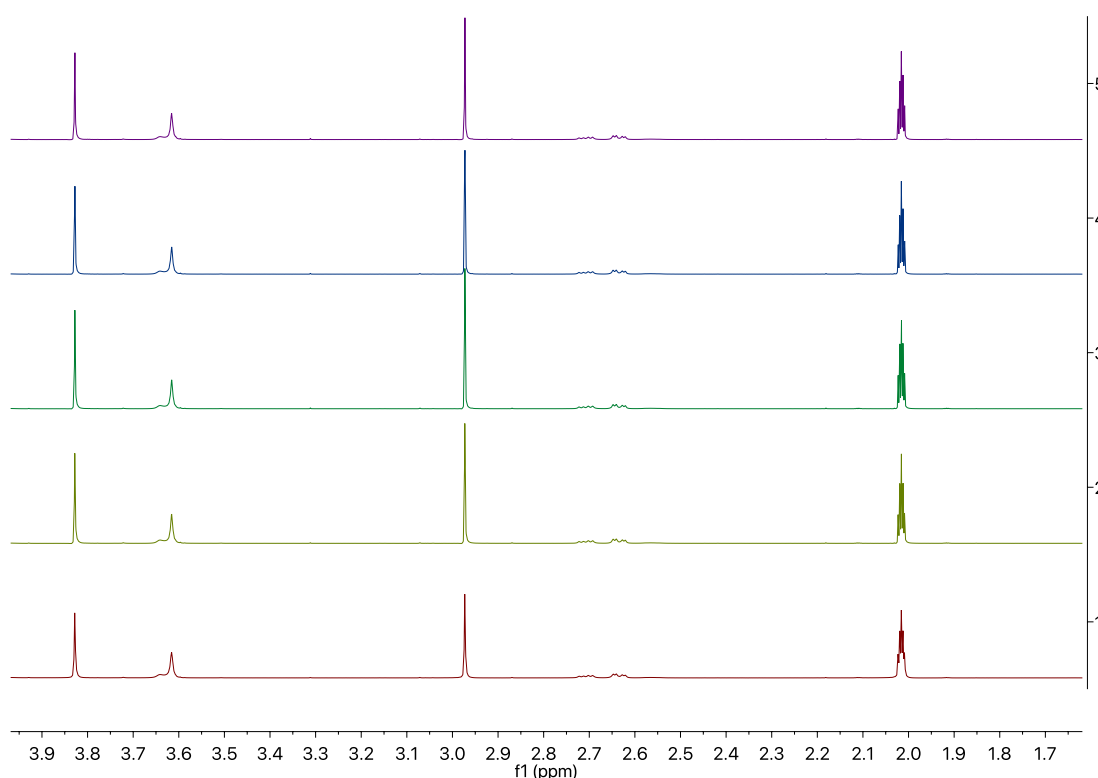
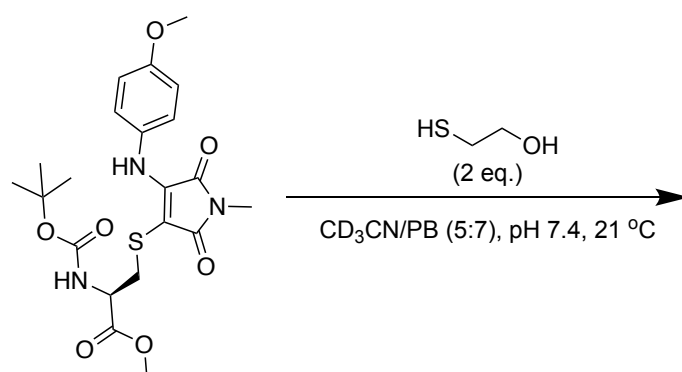


Figure S23: Time course NMR study of **11** in buffer at pH 7.4.

<u>NMR spectra number</u>	<u>Timepoint</u>
1	T=0
2	24 h
3	70 h
4	76 h
5	147 h

Thiol stability



Aminothiomaleimide **11** (4.30 μ mol, 500 μ L, 8.60 mM in CD₃CN) was added to 2-mercaptoethanol (8.60 μ mol, 700 μ L, 12.28 mM in PB (100 mM phosphates), 2 equiv.). The pH was corrected to 7.4 and the solution was incubated at 21 °C for 7 days. The solution was analysed by NMR. The *N*-me peak at 2.98 ppm was monitored carefully to observe if a reaction occurred. No reaction was observed.

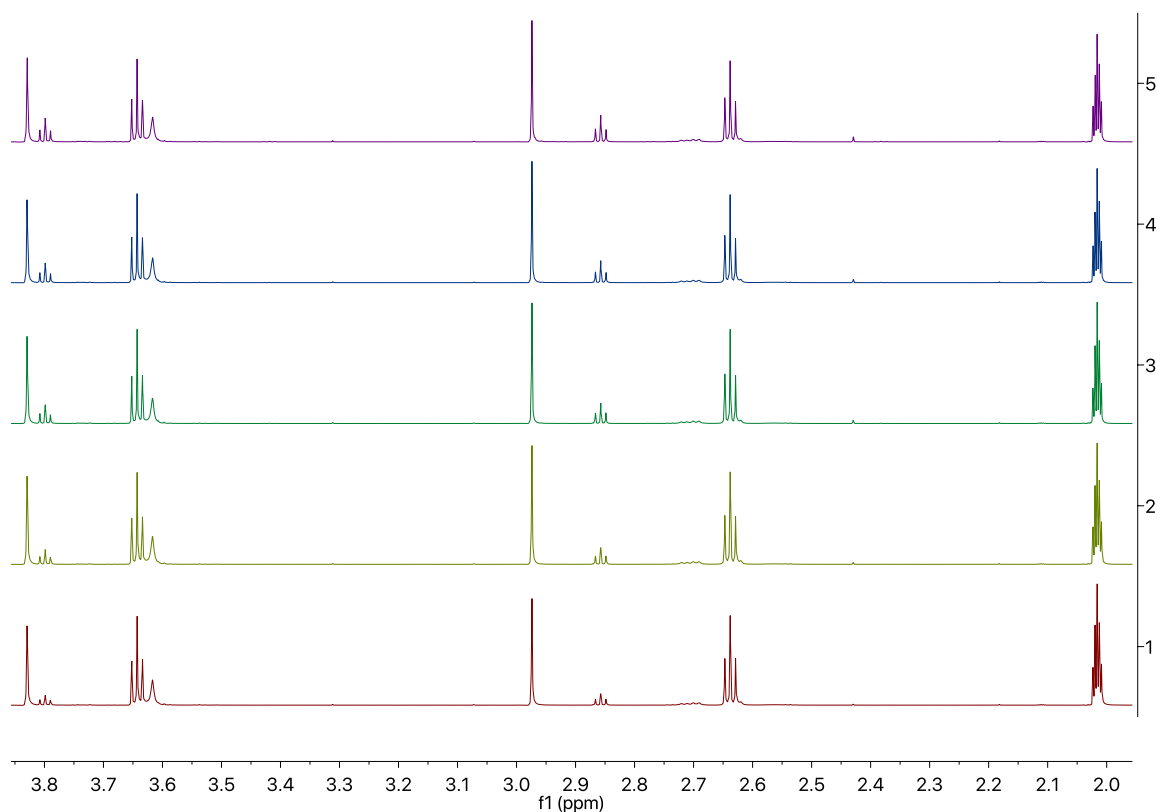


Figure S24: Time course NMR study of **11** in buffer with 2-mercaptoethanol (BME) at pH 7.4.

<u>NMR spectra number</u>	<u>Timepoint</u>
1	19 h
2	43 h
3	66 h
4	72 h
5	7 days

Control vs T5 NMR

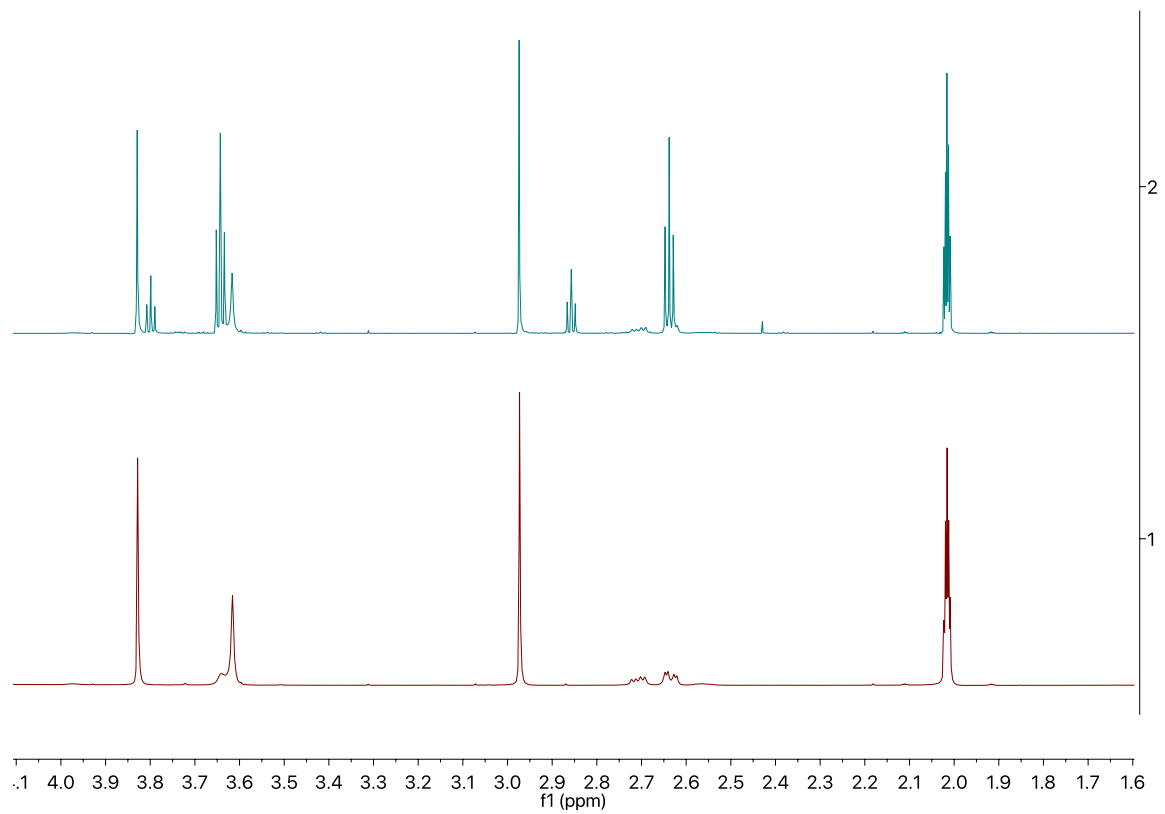
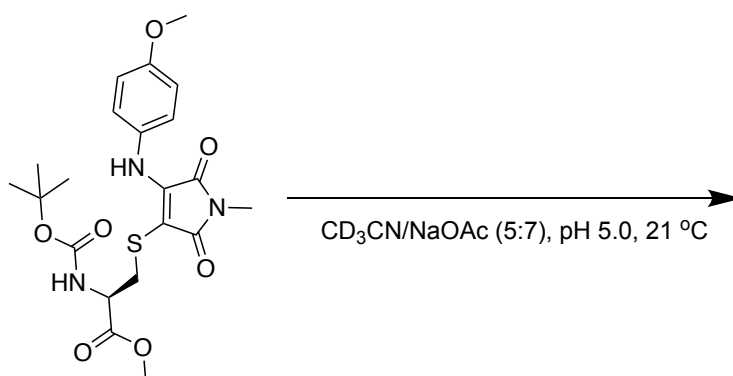


Figure S25: Stacked NMR spectra to show control vs 7 day timepoint after BME addition.

<u>NMR spectra number</u>	<u>Timepoint</u>
1	Control (No BME added)
2	7 days

Buffer stability (pH 5)



Aminothiomaieimide **11** (2.15 μmol , 500 μL , 4.30 mM in CD_3CN) was added to NaOAc buffer (700 μL , 100 mM). The pH was corrected to 5.0 and the solution was incubated at 21 °C for 8 days. The solution was analysed by NMR. The *N*-Me peak at 2.97 ppm was monitored carefully to observe if a reaction occurred. No reaction was observed.

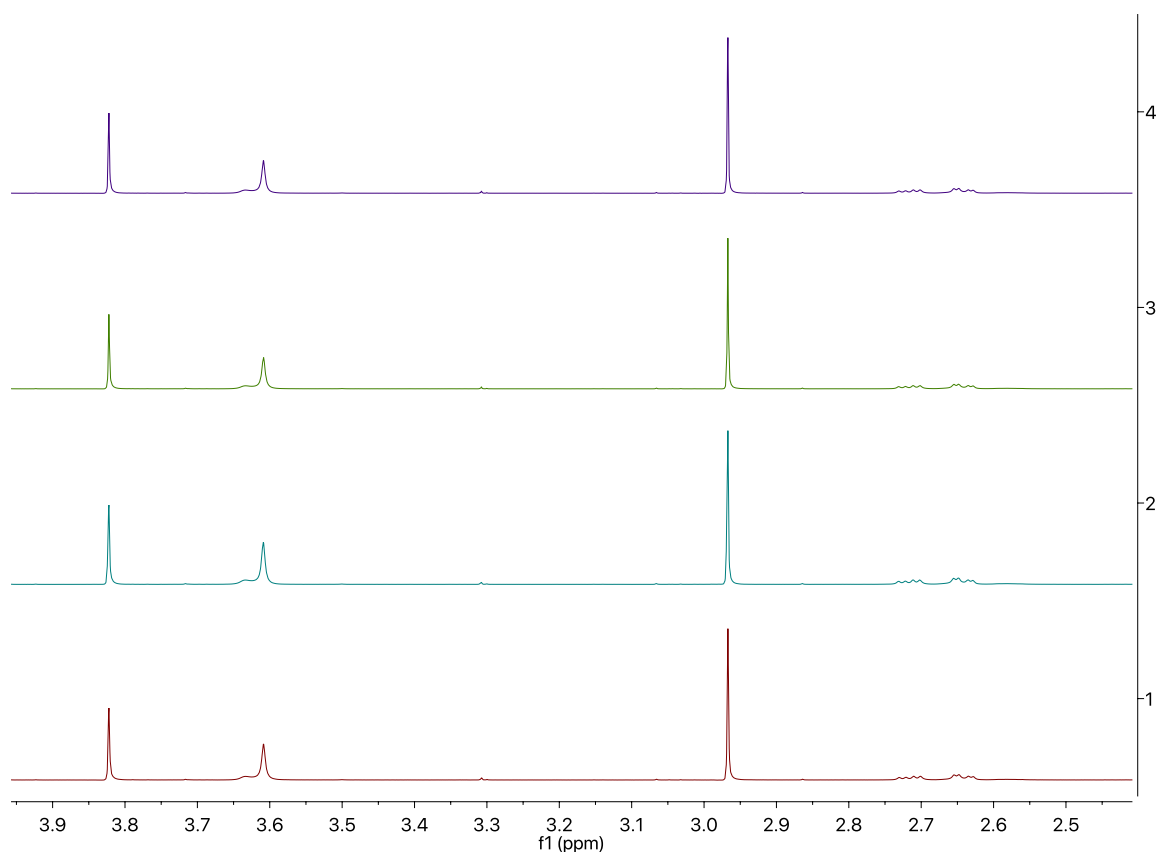
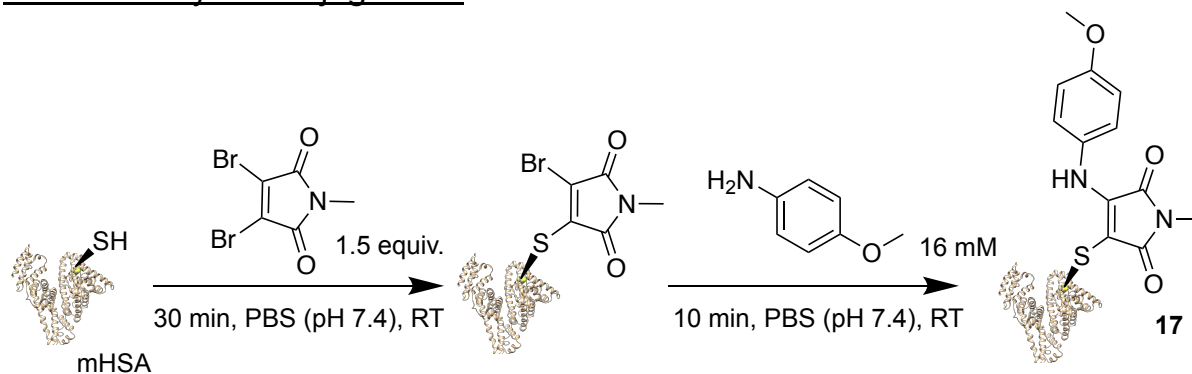


Figure S26: Time course NMR study of **11** in buffer at pH 5.0.

<u>NMR spectra number</u>	<u>Timepoint</u>
1	T=0
2	2 days
3	5 days
4	8 days

Thiol Stability of Conjugate **17**



Bromomaleimide reagent **13** (0.9 μ L, 10 mM, 1.5 equiv. in DMSO) was added to mHSA (96 μ M, 60 μ L) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 30 minutes, *p*-anisidine (1 μ L, 1 M in DMSO) was added, and the reaction left at room temperature for a further 10 minutes. Excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were analysed by LCMS before use in stability assays described below.

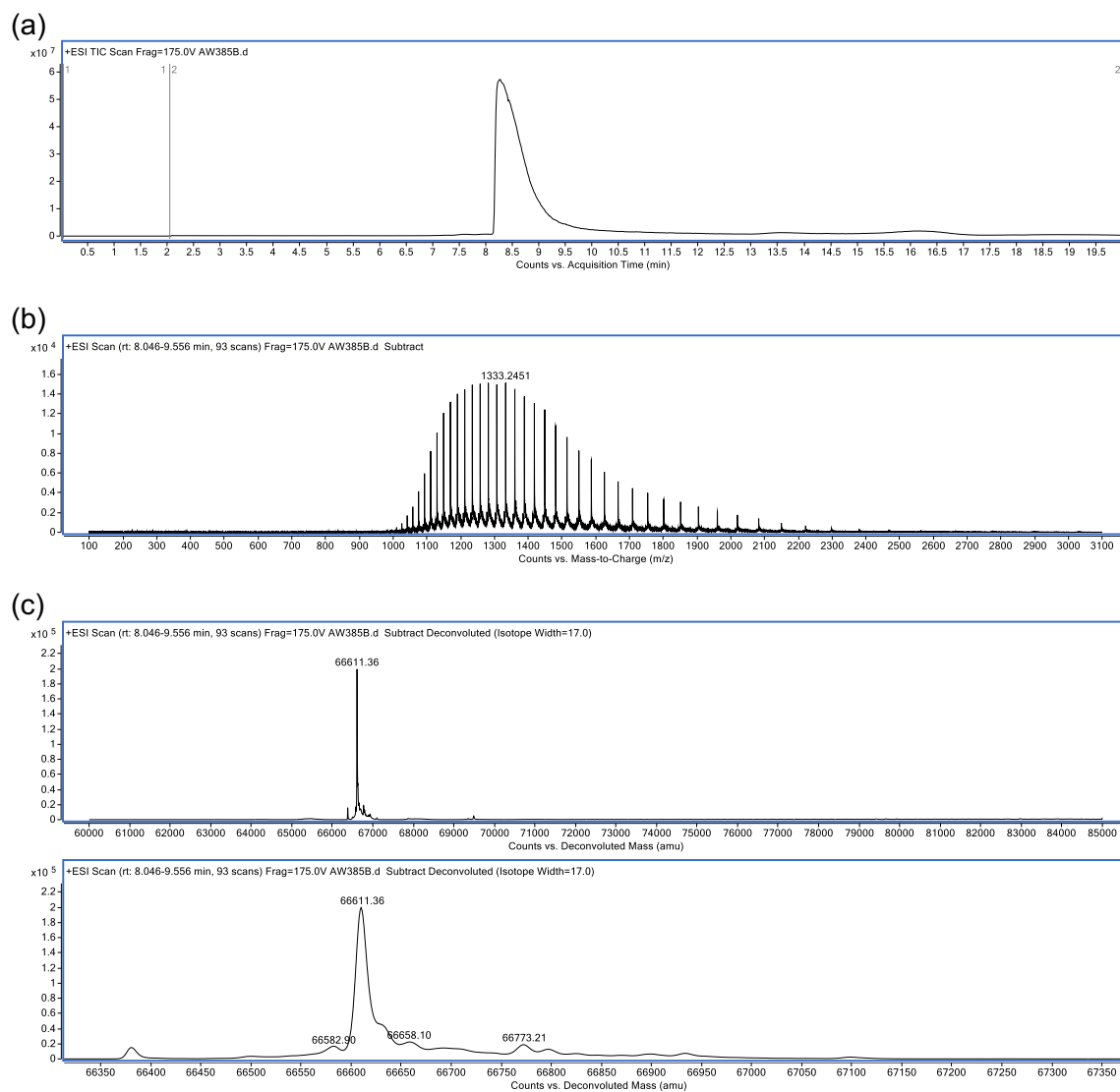
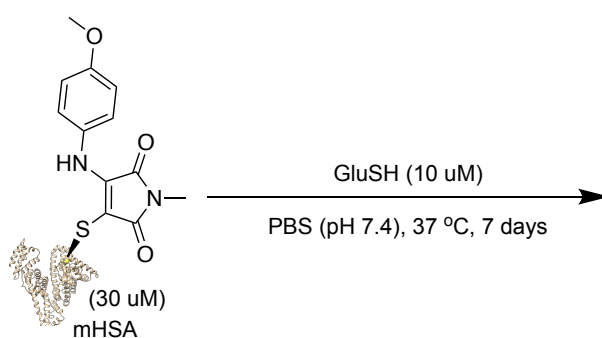


Figure S27: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for *N*-Me DBM **13** conjugated to mHSA with subsequent addition of *p*-anisidine. Expected mass: 66612 Da. Observed mass: 66611 Da. Data obtained with MS method B.

Extracellular Thiol Concentration and pH



Conjugate **17** (30 μ M) was incubated in a solution of GluSH (10 μ M in PBS) at pH 7.4 at 37 $^{\circ}$ C. Timepoints were taken over 7 days and analysed by LCMS. The T0-T4 timepoint data is shown below. Expected conjugate mass: 66,612 Da.

T0 – Before GluSH addition

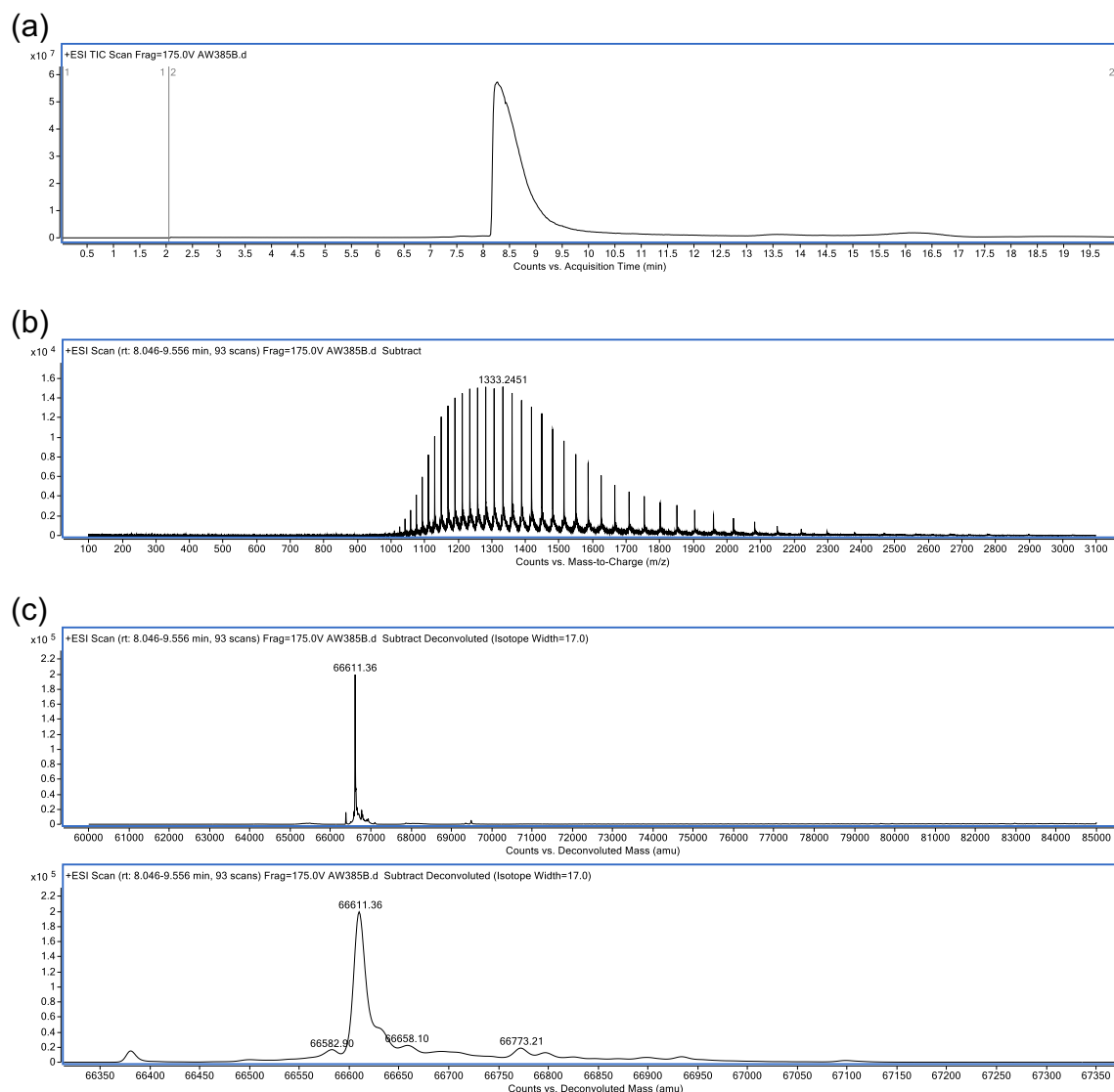
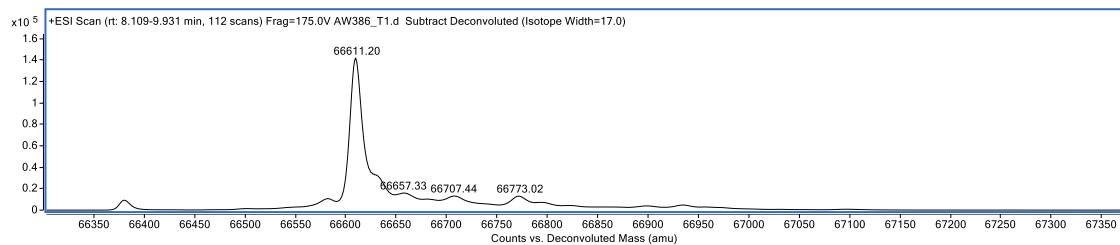
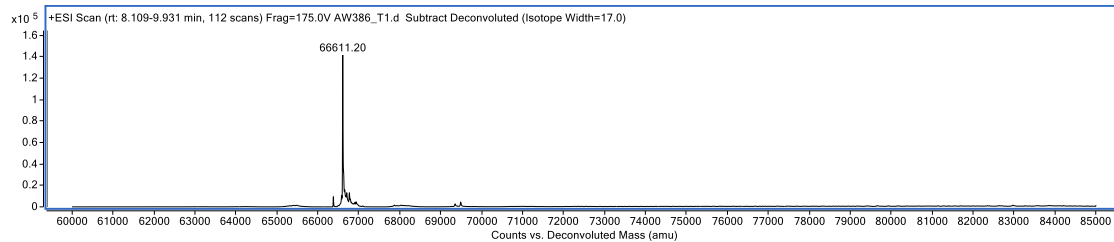
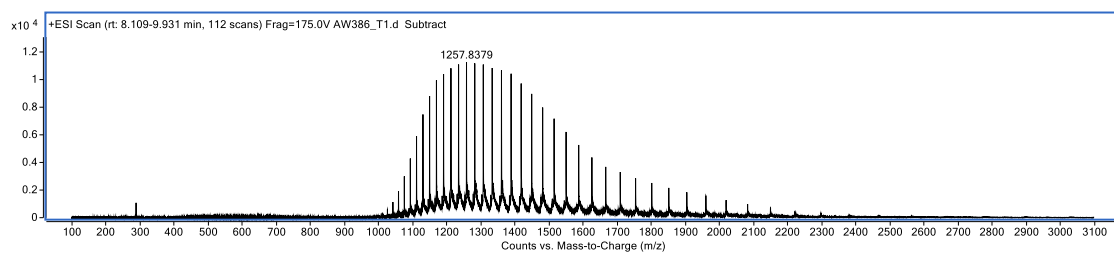
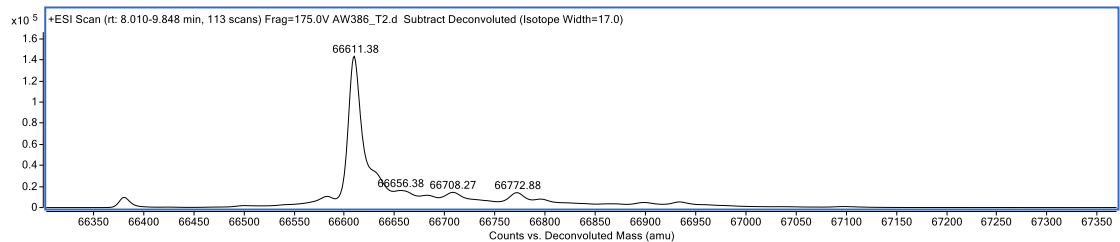
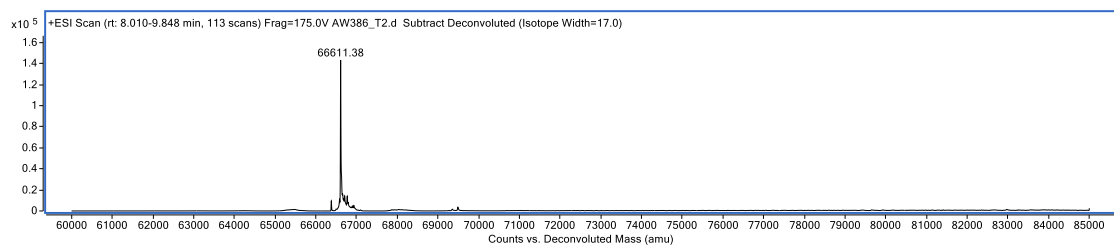
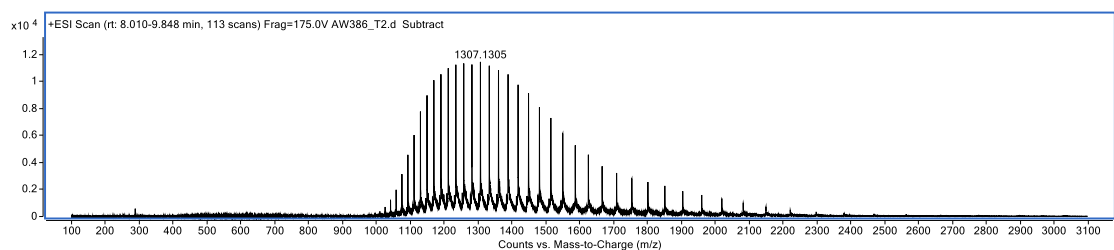


Figure S28: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **17**. Expected mass: 66612 Da. Observed mass: 66611 Da. Data obtained with MS method B.

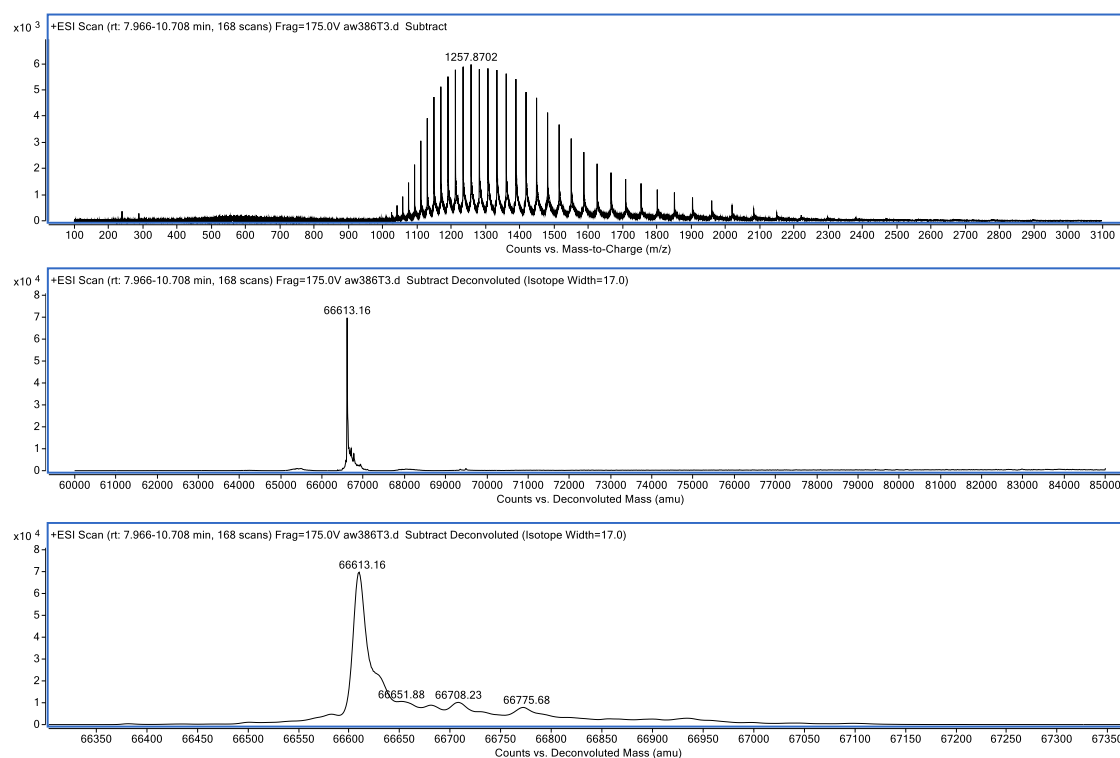
T1 – 24 h post GluSH addition



T2 – 48 h post GluSH addition



T3 – 6 days post GluSH addition



T4 – 7 days post GluSH addition

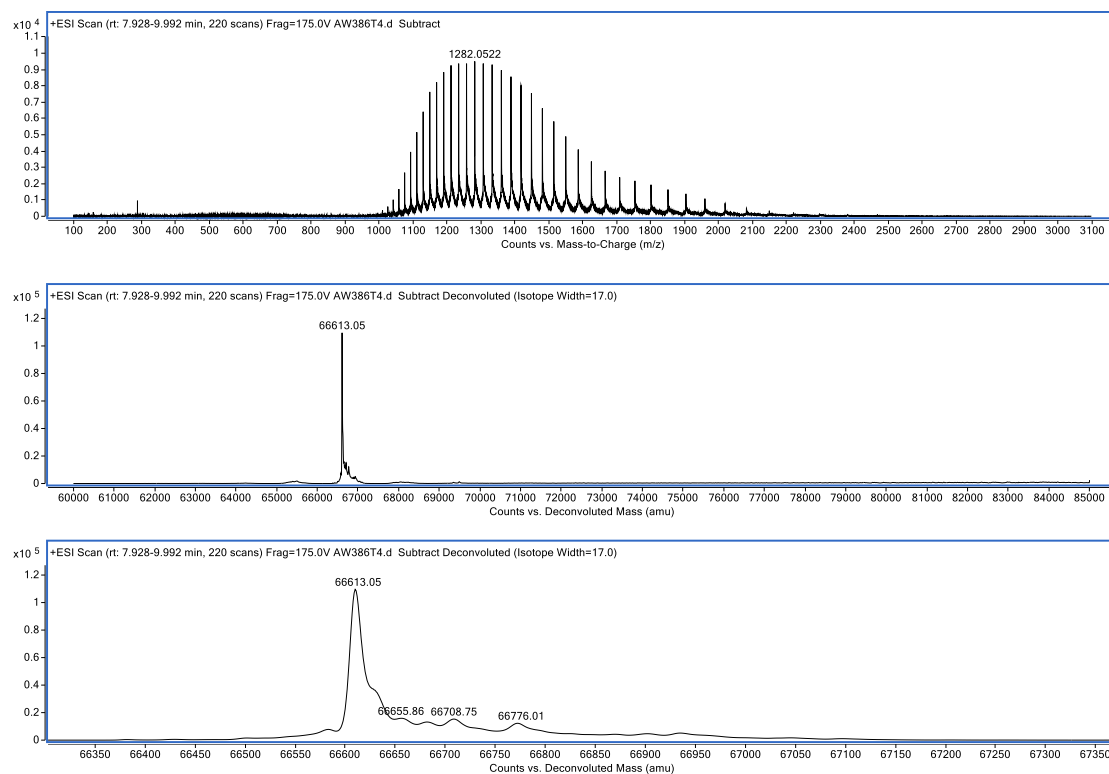
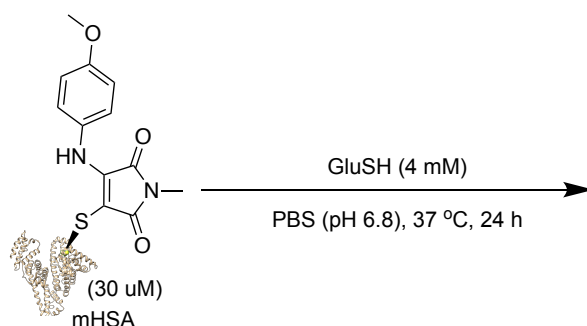


Figure S29: (a) non-deconvoluted data and (b) deconvoluted data for incubation of conjugate 17 with GSH (10 μ M, pH 7.4, 37 $^{\circ}$ C, 7 days). Expected mass: 66612 Da. Observed mass: 66613 Da. Data obtained with MS method B.

Intracellular Thiol Concentration and pH



Conjugate **17** (30 μ M) was incubated in a solution of GluSH (4 mM in PBS) at pH 6.8 at 37 $^{\circ}$ C. Timepoints were taken over 24 h and analysed by LCMS. The T0-T2 timepoint data is shown below. Expected conjugate mass: 66,612 Da.

T0 – Before GluSH addition

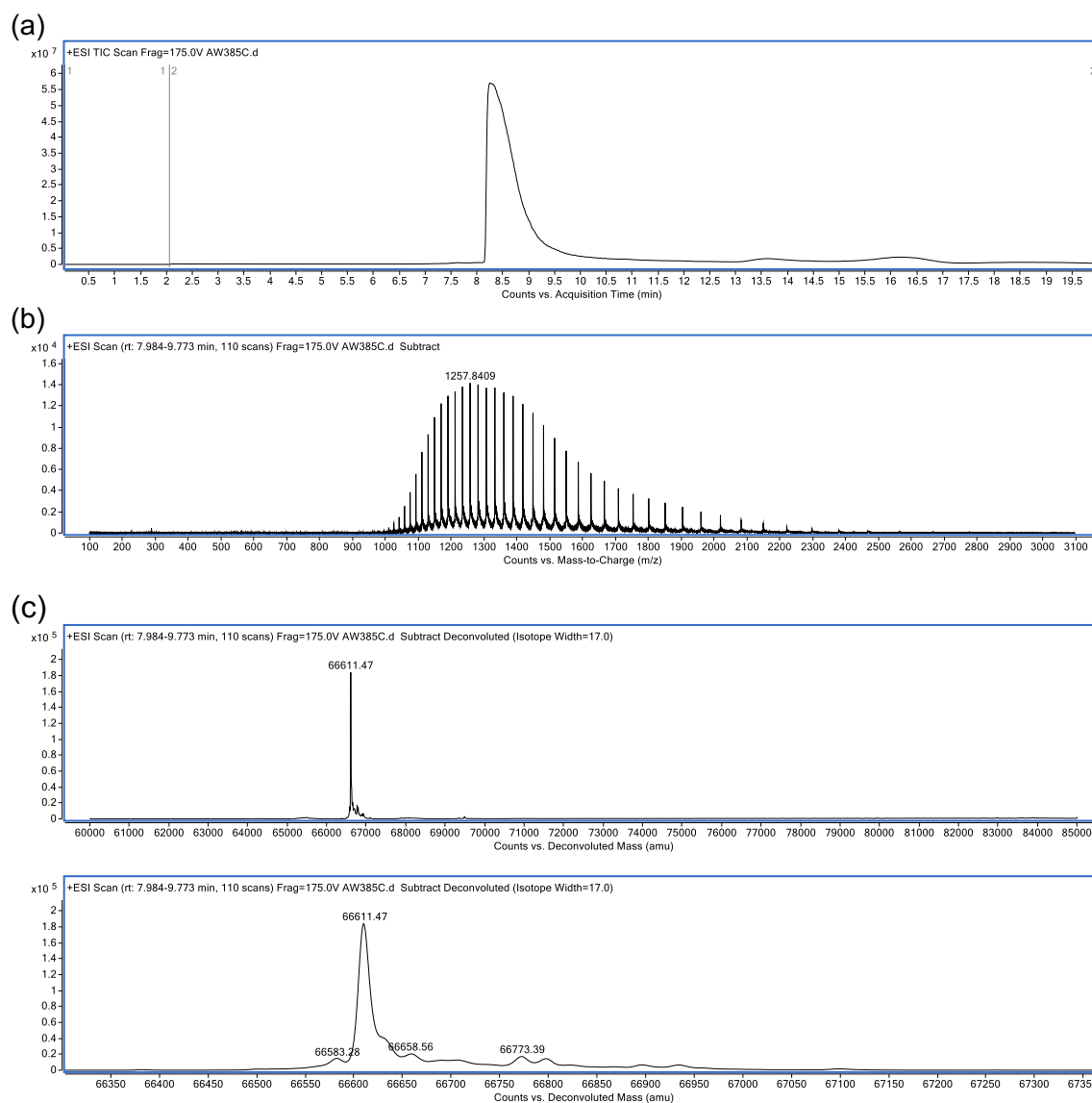
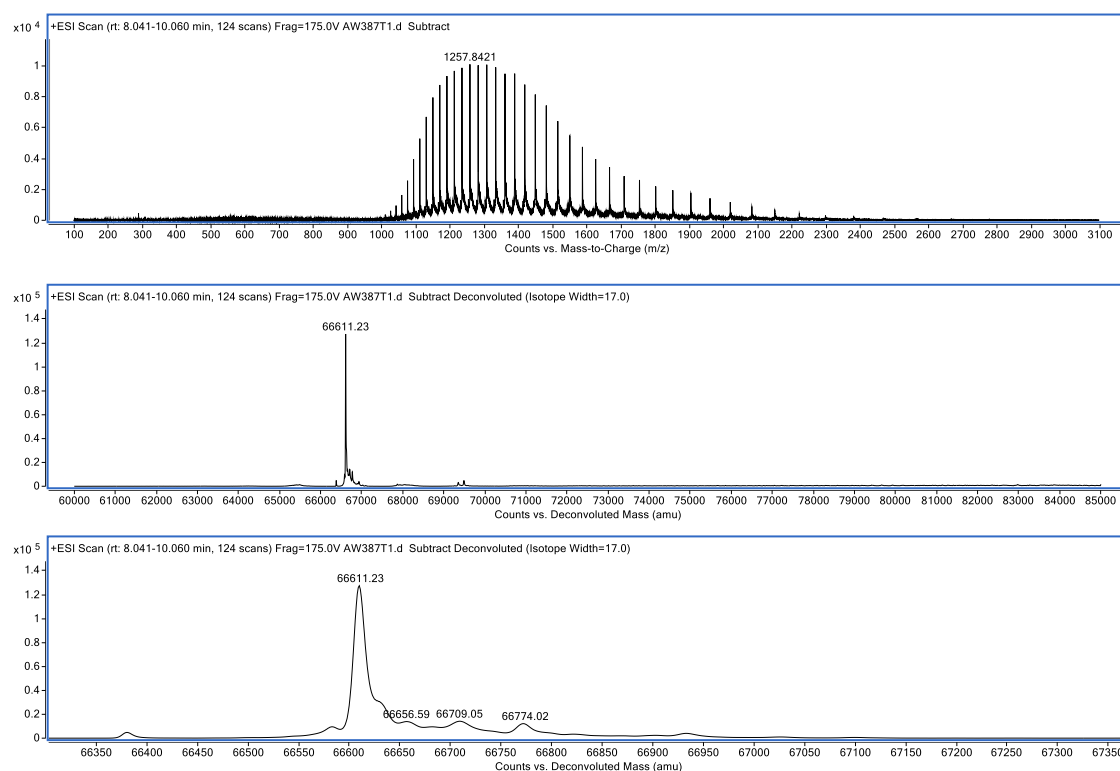


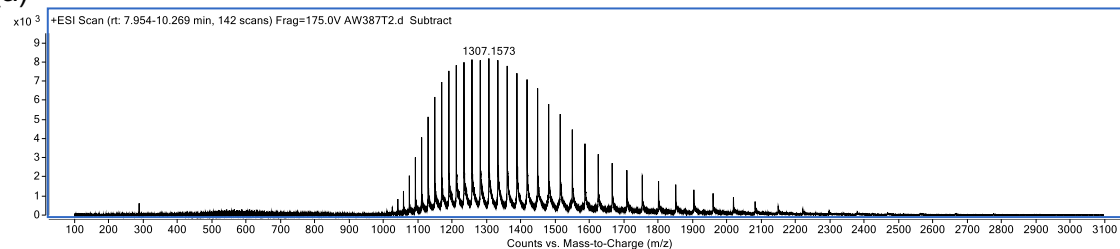
Figure S30: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **17**. Expected mass: 66612 Da. Observed mass: 66611 Da. Data obtained with MS method B.

T1 – 3 h post GluSH addition



T2 – 24 h post GluSH addition

(a)



(b)

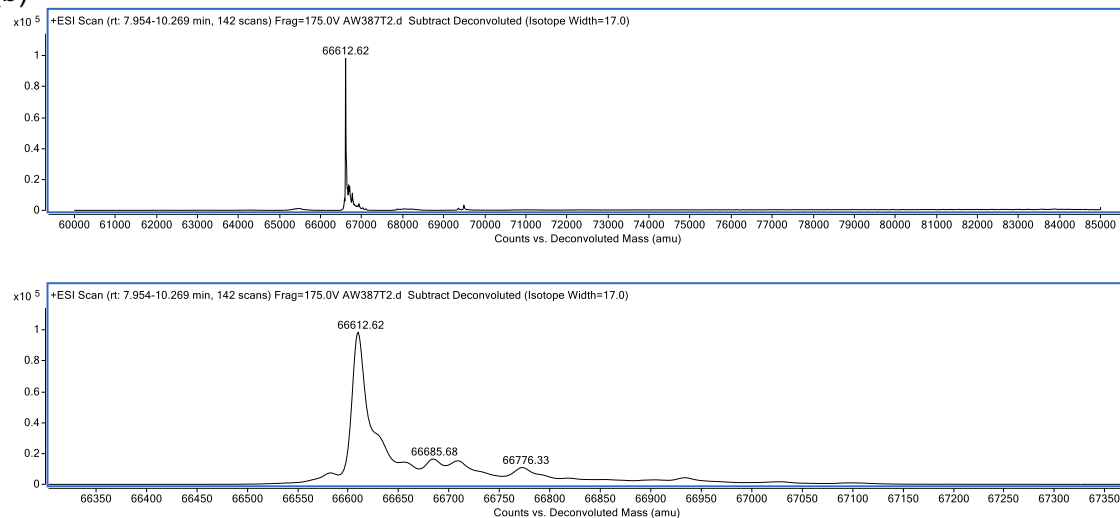


Figure S31: (a) non-deconvoluted data and (b) deconvoluted data for incubation of conjugate **17** with GSH (4 mM, pH 6.8, 37 °C, 24 h). Expected mass: 66612 Da. Observed mass: 66613 Da. Data obtained with MS method B. Small peak at 66686 Da could be tentatively assigned as HSA-GSH dimer although this is unconfirmed.

References

- 1 K. K. Schelde, K. Nicholls, F. Dagnæs-Hansen, K. Bunting, H. Rawsthorne, B. Andersen, C. J. A. Finnis, M. Williamson, J. Cameron and K. A. Howard, *J. Biol. Chem.*, 2019, **294**, 3735–3743.
- 2 L. Castañeda, Z. V. F. Wright, C. Marculescu, T. M. Tran, V. Chudasama, A. Maruani, E. A. Hull, J. P. M. Nunes, R. J. Fitzmaurice, M. E. B. Smith, L. H. Jones, S. Caddick and J. R. Baker, *Tetrahedron Lett.*, 2013, **54**, 3493–3495.
- 3 M. Dubernet, V. Caubert, J. Guillard and M. C. Viaud-Massuard, *Tetrahedron*, 2005, **61**, 4585–4593.
- 4 K. Zhang, J. Liu, S. Ohashi, X. Liu, Z. Han and H. Ishida, *J. Polym. Sci. Part A Polym. Chem.*, 2015, **53**, 1330–1338.
- 5 K. M. Bonger, R. J. B. H. N. van den Berg, L. H. Heitman, A. P. IJzerman, J. Oosterom, C. M. Timmers, H. S. Overkleeft and G. A. van der Marel, *Bioorganic Med. Chem.*, 2007, **15**, 4841–4856.
- 6 K. Dao, R. R. Sawant, J. A. Hendricks, V. Ronga, V. P. Torchilin and R. N. Hanson, 2012, **23**, 785–795.
- 7 G. Burgy, T. Tahtouh, E. Durieu, B. Foll-Josselin, E. Limanton, L. Meijer, F. Carreaux and J. P. Bazureau, *Eur. J. Med. Chem.*, 2013, **62**, 728–737.
- 8 L. Boddu, V. Potlapati and N. J. P. Subhashini, *J. Heterocycl. Chem.*, 2019, **56**, 3197–3205.
- 9 W. Zhang, Z. Li, M. Zhou, F. Wu, X. Hou, H. Luo, H. Liu, X. Han, G. Yan, Z. Ding and R. Li, *Bioorganic Med. Chem. Lett.*, 2014, **24**, 799–807.
- 10 P. Moody, M. E. B. Smith, C. P. Ryan, V. Chudasama, J. R. Baker, J. Molloy and S. Caddick, *ChemBioChem*, 2012, **13**, 39–41.
- 11 R. L. Levine, J. Moskovitz and E. R. Stadtman, *IUBMB Life*, 2000, **50**, 301–307.

Amine-Thiol Dual Conjugation ESI.pdf (27.50 MiB)

[view on ChemRxiv](#) • [download file](#)
