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Development of Novel Maleimide Reagents for Protein Modification

By
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Declaration

I, Cristina Marculescu, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Chemical methods for protein modification are of utmost importance as they allow the mimicking of post-translational modifications that occur in living cells. In addition, they allow exogenous modifications that are the basis for protein enrichment (e.g. biotin labelling), structural analysis (e.g. FRET based studies) and visualisation studies (e.g. fluorescent tagging). Furthermore, chemical modification of proteins enables the synthesis of protein-drug conjugates and the tuning of drug pharmacokinetics.

Bromomaleimides represent the first of a new class of reagents that can be efficiently used for the *fast*, *highly selective* and *reversible* either by addition to a single cysteine or by bridging of disulphide bonds. In order to generate analogous reagents with potentially improved properties, a library of novel analogues was synthesised, each bearing different leaving groups on the double bond of the maleimide. For each, the thiol reactivity, water-solubility, and cross-reactivity with reducing agents were assessed using a single cysteine mutant of the Grb2 SH2 protein. Disubstituted analogues were tested as disulphide bridging reagents using the small model peptide somatostatin. Phenoxymaleimides were investigated as particularly interesting reagents of attenuated reactivity. They were found to efficiently form a succinimide bridge across the disulphide bond of somatostatin. This novel succinimide bridging was further demonstrated on the octoretide peptide, on Fab and scFv antibody constructs. Minimal cross-reactivity of phonoxymaleimides with TCEP enabled addition of TCEP after addition of phenoxymaleimide, which is believed to reduce the risk of disulphide scrambling.
Furthermore, the formation of the succinimide bridge was found to be reversible and the resultant released cysteine could be trapped with a maleimide or bromomaleimide. The result was a dual labelled protein conjugate. This represents a novel method for the dual labelling of disulphide containing biomolecules, allowing the preparation of various dual labelled somatostatin conjugates.

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I dedicate this thesis to my beloved dad and grandma, for all the love and confidence which they gave me and for doing their very best to help me be happy. They are the ones that shaped me as the person I am today so I am grateful to them for everything I have.

Abbreviations

µg	Microgram
µL	Microliter
µm	Micrometre
µM	Micromolar
A	UV-Absorbance
ADC	antibody-drug conjugate
Ar	Aromatic
Aq.	Aqueous
Boc	*tert*-Butyl carbamate
CEA	Carcinoembrionic antigen
$CHCl_3$	Chloroform
CH_3CN	Acetonitrile
CI	Chemical Ionisation
cm	Centimetre
Cys	Cysteine
Da	Dalton
CH_2Cl_2	Dichloromethane
DMAP	4-Dimethylaminopyridine
EDC·HCl	*N*-(3-Dimethylaminopropyl)-*N'*-ethyl carbodiimide hydrochloride salt
EDTA	ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
Et_2O	Diethyl ether
EtOH	Ethanol
Eq.	Equivalent
DMF	Dimethylformamide
DIPEA	*N,N*-Diisopropylethyleneamine
DTT	1,4-dithiothreiol
EI	Electron ionisation
ES	Electrospray
Fab	antigen-binding fragment of an antibody
Fc	Crystallisable fragment of an antibody
FRET	Förster resonance energy transfer

Fv	Variable fragment of an antibody
g	Gram
GSH	Reduced glutathione
H	Hour
HBTU	*O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOAc	Acetic acid
HOBt	1-Hydroxybenzotriazole
HPLC	high-performance liquid chromatography
IR	Infrared
LC-MS	Liquid chromatography-mass spectroscopy
kDa	Kilodalton
kV	Kilovolt
m	Meta
M	Molar
mL	Millilitre
mM	Millimolar
mmol	Millimol
m/z	Mass per charge
m-CPBA	*meta*-Chloroperoxybenzoic acid
m.p.	Melting point
min	Minute
MW	Molecular weight
MWCO	Molecular weight cut off
n	Normal
nm	Nanometre
NMR	Nuclear magnetic resonance
OMe	Methoxy
p	Para
PDB	Protein data bank
PE	Petroleum ether
PEG	Poly(ethylene glycol)
ppm	Parts per million

rpm	Rotation per minute
sat.	Saturated
scFv	Single-chain scFv
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCEP	*Tris*(2-carboxyethyl)phosphine
tert	Tertiary
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
UCL	University College London
UV	Ultraviolet
V	Volt
w/v	Weight-in-volume
6xHis	6 x Histidine tag

I Introduction

I.1 Introduction to chemical modification of proteins

Proteins are responsible for the majority of functions in living organisms; they act as structural supports, as catalysts, and they control the signalling within and between cells. One of nature's ways to generate functionalised proteins from a limited number of genes is *via* post-translational modifications (PTM), such as acylation, glycosylation, phosphorylation, methylation and ubiquitinylation, which provide versatility to proteins by modulating their properties and functions.[1] All post-translation modifications play key roles in cellular processes. Examples include glycosylation which is involved particularly in cell-cell recognition and signalling; acylation with fatty acids which is key to membrane localization and the regulation of hydrophobic interactions between proteins or proteins and cell membranes; and ubiquitination which often precedes protein degradation.[2]

The need to understand biological processes and the role of proteins within them, and also the utility of proteins in therapeutic agents have triggered a lot of research centered around the proteome. This has been enabled by major developments in bioanalytical techniques such as LC-MS, ELISA, HPLC, micro gel-electrophoresis, tandem MS and high-field NMR.

By chemically modifying proteins, scientists are able to mimic the PTMs that occur inside living cells. This can provide insights into the function of proteins in biological processes, their structure and the properties of their functional groups. Fig. 1 shows the most common approach for probing PTMs, promoted as the 'tagging *via* substrate' approach by Tate *et al*..[2] This strategy involves using a modified substrate, normally labelled with a small chemical handle. The tag must not interfere with the recognition between the substrate and the enzyme. Once the enzymatic reaction occurs and the target undergoes the PTM, a chemical reagent is added which is designed such that it reacts only with the chemical label on the substrate. This reagent is also designed as to allow further purification by affinity and characterisation, for example by containing biotin tags or fluorophores.

Fig. 1. Representation of the 'tagging *via* substrate' approach (image taken from Heal *et. al.*)[2]

Such an approach was employed by Wright *et al.*[3] to investigate the *N*-myristoylation of proteins in *P. falciparum*, the parasite that induces malaria in sub-Saharan Africa, and subsequently prove that *N*-myristoyltransferase represents an antimalarial drug target.

Moreover, chemical modification of proteins (CMP) may be used to achieve exogenous synthetic modifications such as the introduction of fluorescent tags for cell imaging[4–6] or for FRET-based structural studies.[7,8] Spin labels have enabled conformational studies and the development of biosensors[9,10] while affinity tags such as biotin[11,12] allow purification of the protein.

Another area in which CMP is important is therapeutics, for the synthesis of protein-drug conjugates,[13–16] of biosensors[17,18] for diagnosis and for modulating drug pharmacokinetics (e.g. increasing *in vivo* half-life by attaching a PEG chain[19,20]). Oncaspar® (pegaspargase) is just one example of a FDA approved protein based drug whose properties have been successfully improved by pegylation. Unpegylated asparaginase, the active enzyme that is responsible in this case for killing leukaemia cells, induces allergic reactions and has a *in vivo* half-life of 20 h. The commercially available drug contains a 5 kDa PEG chain which is correlated with a significantly decreased adverse immune response and an improved *in vivo* half-life of 357 h.[21]

In order for a reaction to be applicable to proteins, peptides and other biological macromolecules, it needs to fulfil many requirements. Probably the most important of all is to have a high *selectivity* for the targeted amino acid in the presence of many other similar residues in the system. In addition to this, the reaction needs to be *biocompatible*, meaning that it needs to take place under conditions that ensure the stability and retention of the activity of the biomolecules involved in the transformation. This generally means aqueous media with minimal amount of organic solvent, low to ambient temperature and at or near neutral pH.

Furthermore, it is useful if the reaction tolerates the presence of salts and surfactants that are often needed for protein stability. For in cell applications in particular, the reaction is ideally quick and the reagents involved exhibit low toxicity.

The ideal labelling reagents would be highly selective, highly reactive and require minimal amounts of organic solvent in order to dissolve in the reaction mixture. Full conversion of the targeted protein is also desirable to avoid separation of unreacted protein, which can be tedious and generally leads to a reduction in the yield of the transformation. Longer reaction times, the addition of a denaturant to favour a reaction at a less solvent accessible site or of excess reagent to speed up the process are commonly employed to achieve full conversion. The drawback of using excess labelling reagent is in some cases the decreased side chain selectivity and of using a denaturant a greater risk of conformational change of the biomolecule which could result in loss of activity.

The combined requirements of chemoselectivity, speed, stability and toxicity of the labelling reagents makes the development of methods for the selective CMP a significant challenge for chemists and biologists and hampers the application of many chemistry techniques. However, due to the intensive studies that have been conducted in the past couple of decades, we now have access to multiple strategies that allow various specific bioconjugations and even in cell tagging studies, as documented by various reviews that deal with this topic.[22,23]

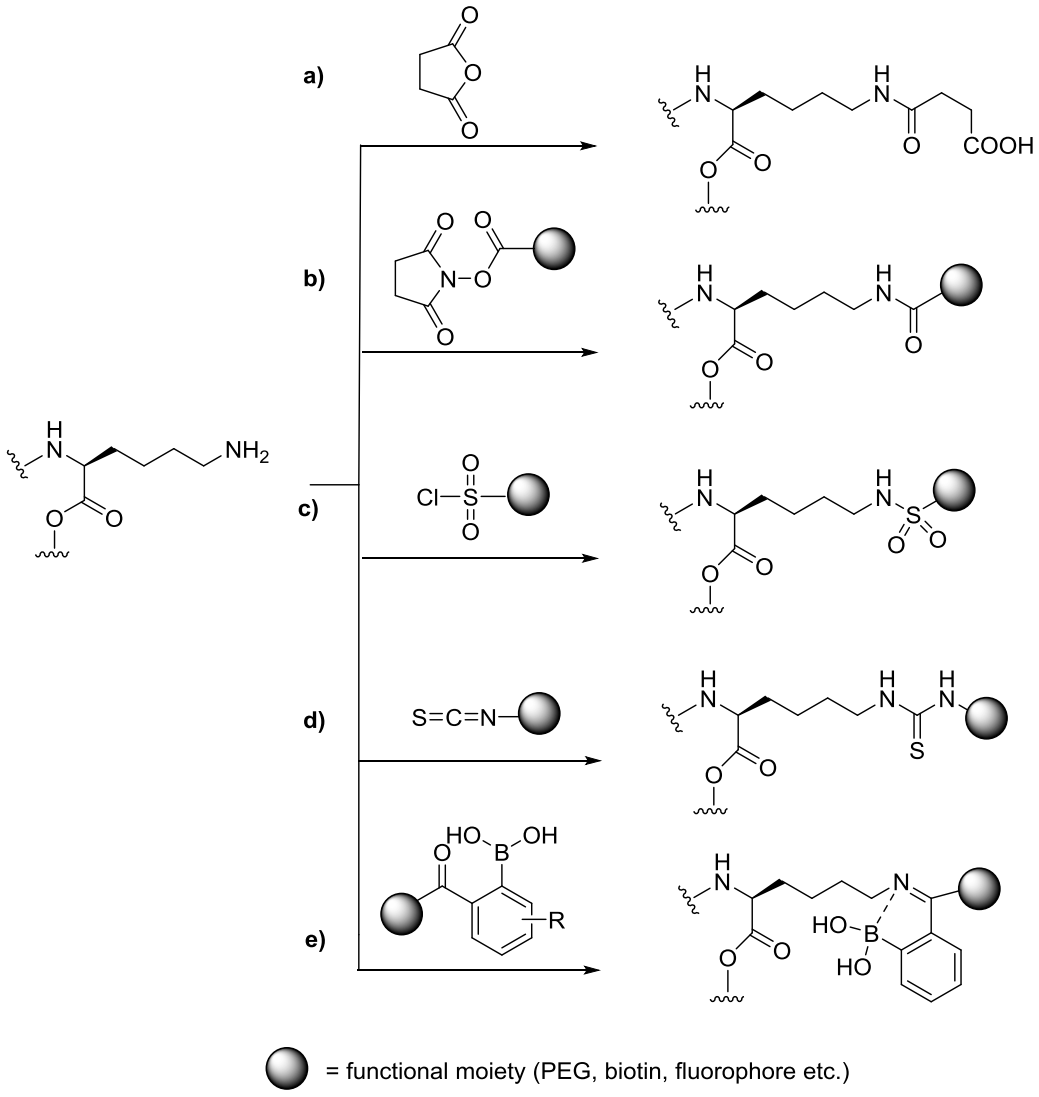
There are two main strategies for achieving CMP: either by a) specifically modifying a native amino acid present in the protein of interest or b) incorporating an unnatural amino acid that contains a bioorthogonal functionality that will serve as point of attachment. This label is ideally inert towards the other groups present in the biomolecule and will solely react with the labelling reagent. Only the former strategy will be considered in the following sections.

I.2 Modification of native amino acids

Although the chemical modification of most of the 21 different naturally occurring amino acids have been reported, the focus has been mostly on lysine and cysteine labelling. This is a consequence of the higher nucleophilicity of these two amino acids compared to the rest, which enables the selective reaction with electrophilic reagents. The following sections will focus on the main strategies employed for their labelling.

I.2.1 Modification of lysine

Lysine residues have received a lot of attention due to their importance in many post-translational modifications and also due to the wide range of chemical methods available to target amino groups. Lysines are labelled at the ε-amino group (pK_a ~ 10.5). The most frequently employed strategy for lysine labelling is acylation with anhydrides (e.g. acetic anhydride,[24] succinic anhydride[25]), activated esters (e.g. *N*-hydroxy succinimidyl esters),[26] sulfonyl chlorides[27] and isothiocyanates[28] (Scheme 1a-d). A recent paper describes the efficient use of iminoboronates in the efficient and reversible lysine modification (Scheme 1e).[29] The imines formed are stabilised by the nitrogen-boron interaction but can be cleaved upon addition of glutathione (GSH).



Scheme 1. Modification of lysine with (a) succinic anhydride, (b) *N*-hydroxy succinimide ester, (c) sulfonyl chlorides, (d) isocyanates and (e) aldehydes

Unfortunately, most of the reagents mentioned above do not react exclusively with lysines, being prone to react with other α-amino groups and also with tyrosine, histidine and cysteine.[30] A common feature of these reactions is that an alkaline pH, generally higher than 8, is required; this is to approach the pK_a of the lysine and therefore ensure that a significant amount of the amino groups are deprotonated (neutral). Together with the high abundance of lysines in proteins, this represents a serious drawback when site-selective modification is required. Generally the reaction outcome is a Gaussian distribution of products and is difficult to direct the labelling onto a specific lysine residue.[31] Lastly, another disadvantage of labelling lysines emerges from the fact that they are positively charged, therefore labelling alters the overall charge of the protein.

I.2.2 Modification of cysteine

In contrast, cysteine has a very low natural abundance (2.26%),[32] and even more, most of the them are tied up in disulphide bonds and therefore are unreactive unless reduced. In addition to that, cysteine is highly nucleophilic and is not charged therefore by labelling with neutral tags the charge of the protein will not change. These features, combined with the possibility of inserting a single cysteine at a chosen location *via* site-directed mutagenesis render cysteine the favourite target for site-directed labelling.

The basis for cysteine modification is that, under physiological conditions, cysteine has a high propensity to form the thiolate anion (pK_a = 8.2). Therefore, it is natural to believe that by using carefully chosen electrophiles, preferential reactivity can be achieved.

At this point, it is important to mention that the situation is far more complex in the context of biological systems as the reactivity of sulfhydryl groups is highly modulated by the local environment. A series of studies have been conducted with the purpose of a better understanding of the effects of factors such as the protein structure, conformation, neighbouring groups and pH on the reactivity of cysteine residues.

To begin with, it is essential to clarify that the reactive species is the thiolate anion (S^-) rather than the thiol (SH) itself.[33,34] This is why the modification of sulfhydryl groups in proteins is optimal when performed at a pH between 7 and 9. Bednar[34] showed in 1990 that the reactivity of the only sulfhydryl group in chalcone isomerase is varies by up to five orders of magnitude with the conformation of the

enzyme. The ionic strength of the solution is another factor that can influence the reactivity of the individual thiols, as stated by Stauffer and Karlin.[35] The residues in proximity to the targeted cysteine group can also exert a significant influence on the pK_a. This is generally valid for proximal cationic groups such as the side chains of lysine or arginine side chains that stabilize the thiolate thus contributing to the depression of the pK_a value of the cysteine.[36,37]

As mentioned before, most cysteines in proteins are found tied up in disulphide bonds, also called cystines (Fig. 2). They are formed during or post-protein folding and are generally responsible for helping to maintain the tertiary structure, afford increased stability and therefore retain the activity of the protein. Reducible disulphides are also involved in the regulation of gene expression and have catalytic activity.[38]

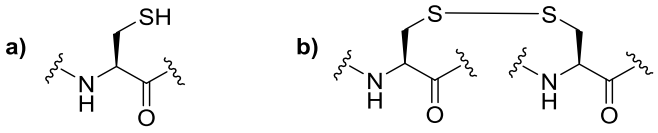


Fig. 2. Structures of (a) cysteine and (b) cystine

The challenge with cystine modification derives from the fact that these residues are responsible for contributing to the preservation of the natural tertiary structure[39–41] of the proteins and that its cleavage leads, in some cases, to the loss of biological activity.[42,43] Engineering an artificial disulphide bond into a protein to increase its stability is common.[44]

In conclusion, the above mentioned features make cysteine a very attractive target when site-selective modification of proteins is required. This is proven by the numerous studies in the area which describe the use of this strategy in the study of protein activity,[45] protein visualisation[46] and for carrying out structural studies.[47]

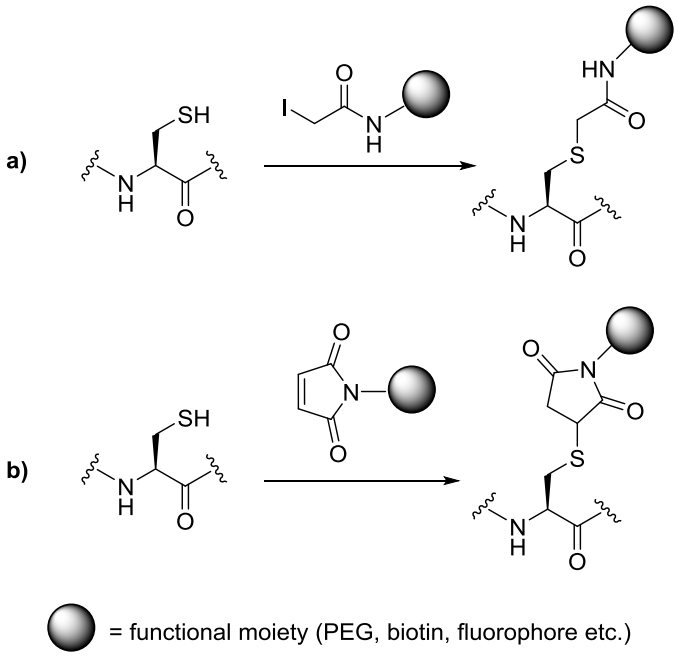
A selection of the most commonly used approaches developed to install selective chemical functionality on cysteine, together with some recent applications will be presented in the following sections.

I.2.2.1 Alkylation of cysteine

From a mechanistic point of view, alkylation of cysteine proceeds *via* S_N2 nucleophilic substitution or *via* conjugate addition.

Alkylation of cysteine by nucleophilic substitution is possible with a wide variety of electrophiles; the first reagents to be reported were the α-halocarbonyl compounds such as iodoacetamides[48] (Scheme 2a) or chloroacetic acid.[49]

Haloacetic acids are generally far slower than the corresponding haloacetamides and by changing the halogen it is possible to slow down or speed up the reaction, depending on the requirement.[50] Among the haloacetamides, the order of reactivity is I > Br >> Cl. These compounds can be easily functionalised allowing the introduction of useful tags in the protein, such as fluorophores,[51,52] spin labels[53] radiolabels[54] and sugars[55]. Despite being characterised by a high cysteine specificity there have been reports of side reactions involving other residues such as histidine, lysine, methionine and tyrosine.[56–59] Furthermore, the slow rate of reaction suggests that these compounds are not compatible with applications when short reaction times are required. This can be overcome by adding excess reagent but this can lead, as mentioned before to unspecific labelling.



Scheme 2. Alkylation of cysteine with (a) iodoacetamide and (b) maleimide

Faster reagents are represented by conjugate acceptors such as maleimides (Scheme 2b),[60] vinyl sulfones[61] and 4-vinyl pyridine.[62,63] In these cases the alkylation proceeds *via* a conjugate addition reaction. The most frequently used reagents are *N*-substituted maleimides, as they react quickly, give no by-products and have high thiol specificity. Also, the nitrogen atom in the ring allows facile functionalisation; many such functionalised maleimide reagents are now commercially available.

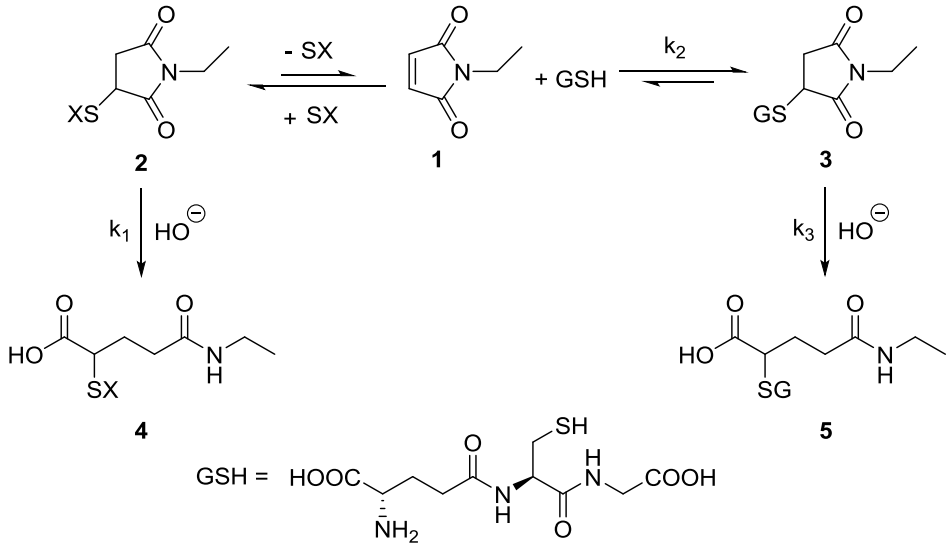
Examples of the application of *N*-substituted maleimides range from performing measurements of thiols in biological fluids,[64] *in vivo* imaging studies,[65,66] structural and functional studies of proteins[67–69] and synthesis of protein-drug

conjugates[70,71] to the development of bioconjugates such as artificial metalloenzymes[72] and biosensors.[73]

In his studies on the reactivity of *N*-ethyl maleimide **1** (Scheme 3) with the amino acid glycylalanine and the the α and β chains of haemoglobin, Smyth[74,75] highlights the risks when using this reagent for specific cysteine targeting. Conditions that favour the specificity towards thiols are a pH below 7 (to reduce the risk of side reactions with amino groups, which at an acidic pH would be protonated, therefore no longer available for nucleophilic attack) and the use of minimal quantities of reagents.

The thiosuccinimide **2** formed in the reaction between a cysteine containing protein or a simple thiol and maleimide is generally stable, limiting its application when temporary modification of proteins is required. However, there are some reports in the literature that describe the degradation of the product in strong reducing environments, such as the cytoplasm of cells[76–78] where the concentration of glutathione can reach 15 mM.[79]

Scheme 3 shows the proposed mechanism for the degradation process observed, together with the hydrolysis that generally accompanies the process. The first step is a retro-Michael addition to regenerate maleimide **1**, which can then react with other thiols in the system, generally glutathione. The equilibrium is significantly shifted to the side of thiosuccinimide **2**. Both the starting material, thiosuccinimide **2** and the product, glutathiosuccinimide **3** can undergo hydrolysis. The resulting thiosuccinic acids **4** and **5** respectively can no longer undergo the retro-Michael addition.



XSH = small thiol or cysteine containing protein

Scheme 3. Degradation pathway of thiosuccinimide **2** in the presence of glutathione

Baldwin *et al.*[80] studied the kinetics of the retro-Michael addition and exchange reactions undergone by thiosuccinimide in reducing environments, highlighting the potential of using this strategy for a controlled release of drugs or degradation of materials. They have found that the formation of **1** from **2** is the rate determining step in the formation of **3**, the addition of glutathione to maleimide being very fast. The paper includes a kinetic study of the thiol-glutathione exchange and concludes that the kinetics of the transformation is significantly dependent on the nature of the thiol substituent (Fig. 3). For example, substituents such as 4-mercaptophenylacetic acid (pK_a = 6.6, blue trace in Fig. 3) will favor the retro-Michael addition while substituents such as 3-mercaptopropionic acid (pK_a = 10.3, green trace in Fig. 3) will give thiosuccinimides that are fully stable under the conditions tested. The thiosuccinimide adducts of thiols with intermediate pK_as, such as the *N*-acetyl-cysteine (pK_a = 9.5, red trace in Fig. 3) will undergo the Michael-addition reaction but at a slower rate compared to the one bearing less acidic substituents.

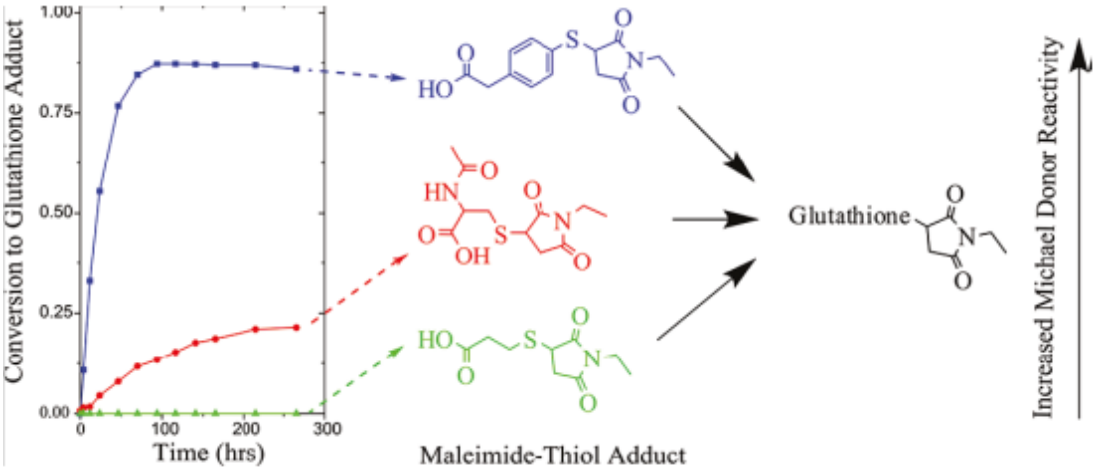


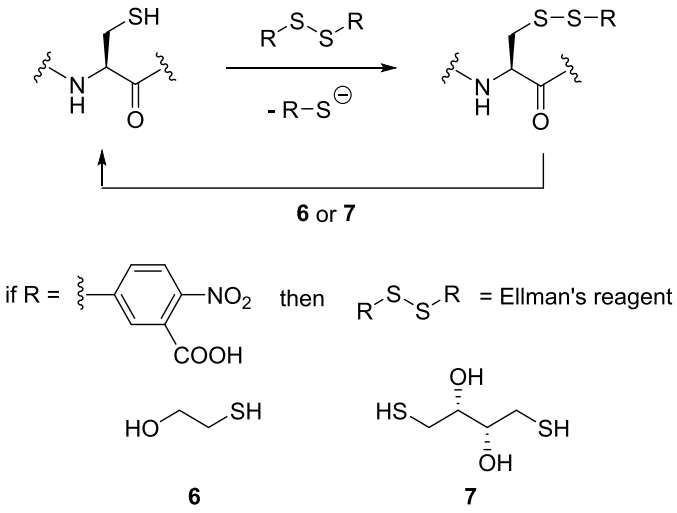
Fig. 3. Reaction profiles for the thiol - GSH exchange for various thiosuccinimides (taken from Baldwin *et al.*)[80]

The same paper highlights the potential use of the thiosuccinimide-based strategy as a drug-delivery strategy complimentary to the disulphide-mediated release, which is a well-established strategy in drug delivery.[81] The time-scales of the two strategies are very different: while the half-lives of disulphides in highly reducing environments (10 mM GSH) are less than 1 h, those of thiosuccinimides are between 20 - 80 h.[80]

In conclusion, the thiosuccinimide-based strategy may find applications in therapeutics, when a slower release of the drug is required. Without disregarding the wide scope of the transformations, the competing hydrolysis reaction reduces its versatility.

I.2.2.2 Oxidation of cysteine

Thiol oxidation is the basis for some post-translational modifications (e.g. *S*-glutathionation)[82] and protein folding.[83,84] Thiol groups react readily with disulphides or selenocysteines, as confirmed by the various disulphide-containing bioconjugates that have been prepared using this method (Scheme 4).[90–92] 5,5'-dithiobis(2-nitrobenzoicacid), known as Ellman's reagent (Scheme 4), is a standard reagent for measuring the concentration of cysteine residues in proteins[88] that has also been used for the modification of cysteine containing proteins such as human[89] and chicken haemoglobin.[90]

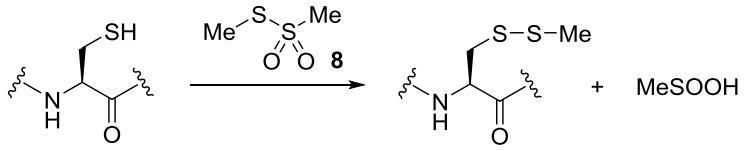


Scheme 4. Reaction of cysteine with disulphides

These transformations can be easily reversed by adding excess of a thiol such as 2-mercaptoethanol **6** and dithiothreitol **7** (DTT). Consequently, disulphides are unstable in the cytoplasm of cells due to the high concentrations of glutathione present there. This feature has turned them into attractive targets as drug delivery vehicles.[91,92]

Disadvantages of these methods include lack of control over the reaction progress and formation of dimers. To address these points, alkyl alkanethiosulphonate reagents have been developed. They react rapidly and selectively with cysteine, providing the corresponding mixed disulphides and sulfinic

acid as the by-product of the reaction. Examples include methyl methanethiosulfonate **8** (Scheme 5) but there are also *bis*-functional reagents such as 1,2-ethanediyl bismethanethiosulfonate (Fig. 4a).



Scheme 5. Disulphide formation from cysteine and methanethiosulfonate **8**

A series of derivatives of the latter (mono-, di- and triacidic acid methanethiosulfonates), (Fig. 4b) were used to investigate the effect of adding negative charges on the activity of the enzyme subtilisin *Bacillus lentus*,[93] while 1,2-ethanediyl bismethanethiosulfonate was used to cross-link and probe conformational changes of *Escherichia coli* AcrBtransporter during its functional cycle.[94]

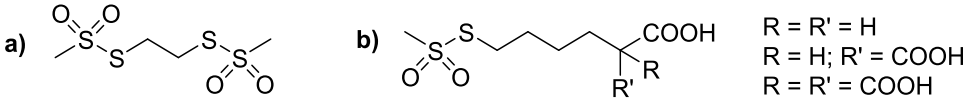


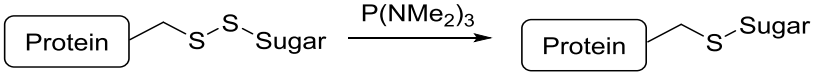
Fig. 4. Examples of *bis*-functional methanethiosulfonates: (a) 1,2-ethanediyl bismethanethiosulfonate; (b) mono-, di- and triacidic acid methanethiosulfonates

I.2.2.3 Desulfurisation at cysteine

a) Disulphide contraction to thioether

The methodology for the desulfurisation of cystine has been developed by Harpp and Gleason.[95] Its importance resides in the fact that it renders the starting disulphide constructs into the corresponding thioethers which, as opposed to disulphides are stable under reducing conditions.

This transformation is achieved by the use of aminophosphines such as $P(Et_2N)_3$ and $P(Me_2N)_3$ (Scheme 6). In their study, Harpp and Gleason investigated the scope and mechanism of the reaction and provided the first example of transformation of a glycosidic disulphide protein conjugate into the corresponding thioether (Scheme 6). This is of valuable importance when considering the biological activity of various thiosugars.

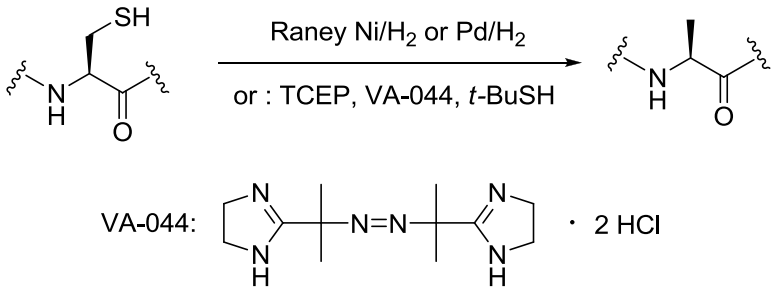


Scheme 6. Disulphide contraction to thioether on a protein substrate

More recent applications based on this strategy come from the Davis[96] group which showed that the strategy is efficient for the synthesis of thioether-linked glycoproteins and glycopeptides.

b) Reductive removal of cysteine thiol

This strategy is outlined in Scheme 7 and consists of a metal-mediated dechalcogenation. As it converts cysteine to alanine, it has gained considerable importance especially after the development of native chemical ligation.[97]



Scheme 7. Desulfurization of cysteine to alanine

Palladium/H_2,[98] nickel/H_2[99] and palladium/Al_2O_3[98] represent standard conditions for achieving this transformation. However these methodologies require a large excess of the metal which can potentially affect other functionalities present in the protein (methionine and the acetamidomethyl (Acm), the latter being a common protecting group). A large excess of the metal can cause epimerization of secondary alcohols and the reduction of other thiols, thioethers and thioesters. Danishefsky *et al.*[100] developed an alternative methodology, based on free radicals derived from combining tris(2-carboxyethyl)phosphine (TCEP), *tert*-butylthiol and a water soluble radical initiator, VA-044 (Scheme 7) which they successfully applied on glycopeptide and polypetide systems. The *tert*-butyl thiol acts as a proton source and while it is not essential for the reaction, it was shown that it speeds up the reaction. Their method is cysteine specific and involves milder conditions.

I.2.2.4 Oxidative elimination of cysteine

The conversion of cysteine and serine to dehydroalanine (Fig. 5) is now a well-established post-translational modification, favoured by exposure to heat and alkali.[101] *In vitro* experiments showed that the oxidative elimination of alkylated derivatives of cysteine and selenocysteine leads to dehydroalanine (Dha). Early methods involved incubating the protein with alkali but this is not recommended due

to side reactions such as the the addition of lysine residues onto the newly formed Dha.[102]

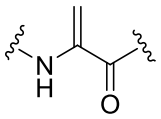
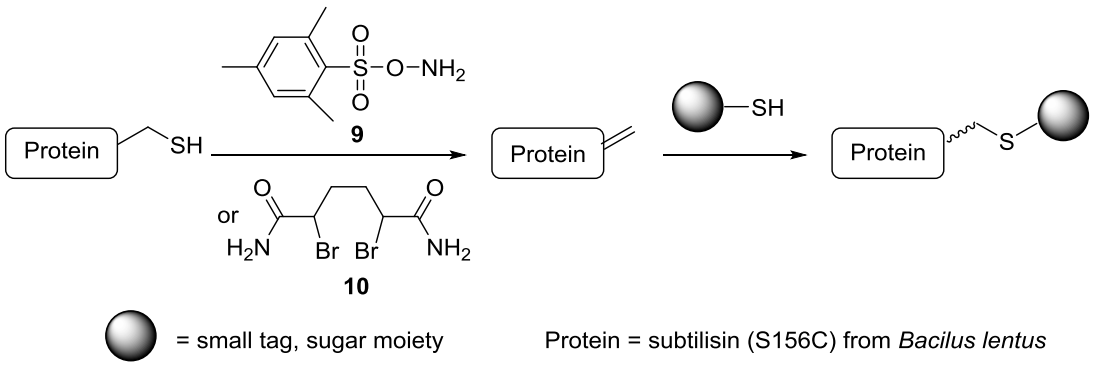


Fig. 5. Structure of dehydroalanine

Bernades *et al.*[103] have reported on *O*-mesytilenesulfonylhydroxylamine **9** (MSH) as a useful reagent for the fast, selective and efficient modification of cysteine residues *via* Dha (Scheme 8). Their strategy is based on the formation of Dha when cysteine is treated with excess MSH under basic conditions. The Dha moiety can then be reacted with thiol nucleophiles to give the corresponding thioethers (Scheme 9). The strength of this method was proven on a single cysteine mutant of the subtilisin *Bacilus lentus* (S156C) serine protease as model system; it was shown that the method allows the fast and efficient introduction of tags that correspond to post-translational modifications such as phosphorylation, glucosylation, farnesylation and *N*-methylation/acylation of lysines. No cross-reactivity with other amino acids was observed. The same group reported on the efficient use of dibromodiamine **10** (DBDA) for Dha formation (Scheme 9). They exemplified the utility of this method in a study on the synthesis of various histones bearing diverse post-translational modifications.[104]



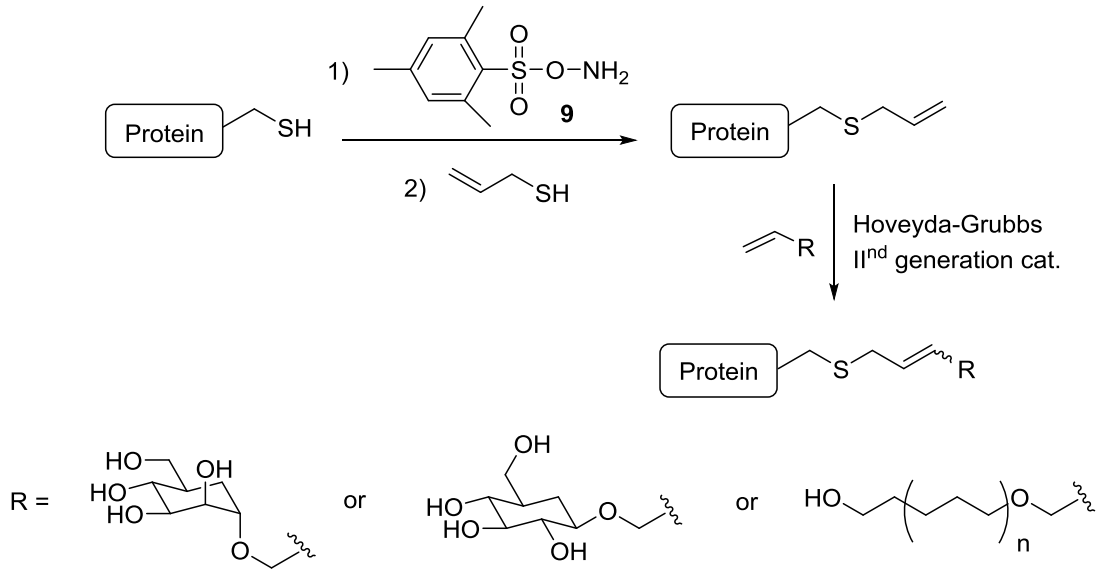
Scheme 8. Oxidative elimination of cysteine to dehydroalanine and further functionalisation with thiol species on a protein substrate

I.2.2.5 Metal-mediated modifications at cysteine and derivatives

One powerful method for cysteine modification which has emerged in recent years is olefin metathesis. For a long time, this type of chemistry was inaccessible to chemical biologists as the methodology was not suitable for aqueous systems. The

development of water-soluble catalysts triggered extensive studies on the application of this methodology on biological systems.

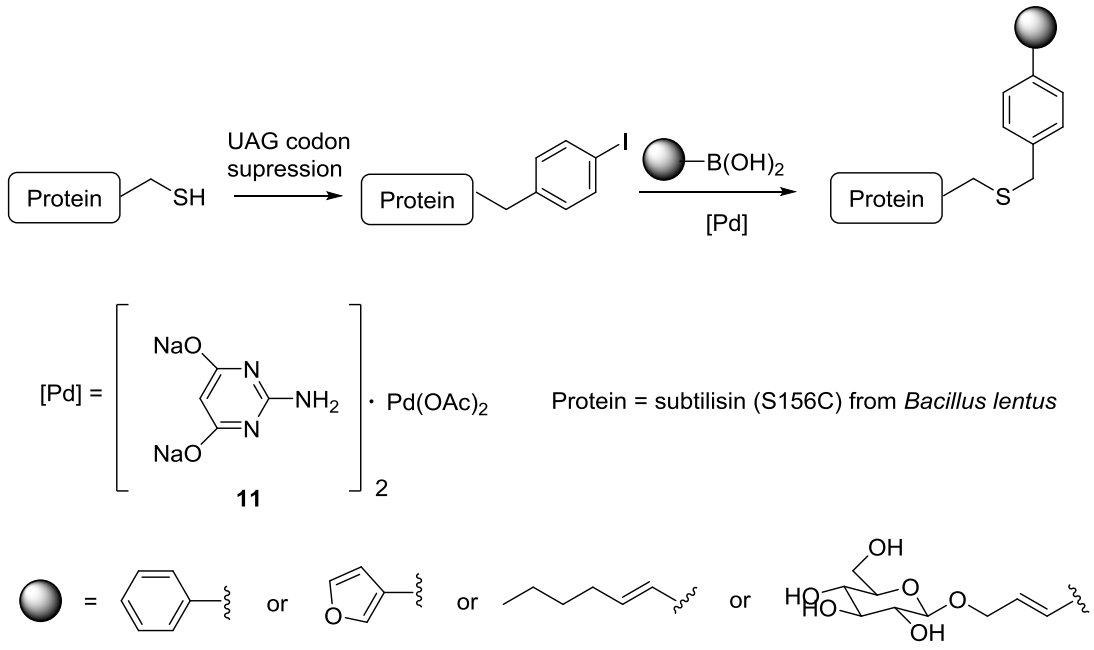
The Davis[105,106] research group is one of the promoters of this strategy. Their strategy takes advantage of the enhanced reactivity of allyl sulphides in cross metathesis. The first step is the incorporation of an allyl sulphide (and more recently the allyl selenide) moiety on the cysteine *via* Dha formation, which is followed by the cross metathesis step (Scheme 9). The methodology proved successful for a very wide range of alkane substrates, including functionalised sugars. This set of examples shows the potential of this strategy to allow easy access to glycopeptides.



Protein = subtilisin (S156C) from *Bacilus lentus*

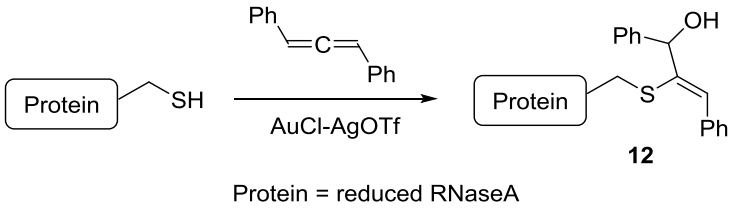
Scheme 9. Olefin metathesis at *S*-allyl cysteine on a protein substrate

Another emerging strategy involves palladium catalysed cross-coupling reactions, mainly the Suzuki-Miyaura coupling. In 2009, Chalker *et al.*[107] reported on the novel water-soluble catalyst **11** that provided higher conversions compared with previously reported attempts to apply this reaction to proteins. The strategy requires the incorporation of a *p*-iodophenylalanine *via* UAG amber codon suppression; this residue will then undergo a Suzuki-Miyaura reaction in the presence of the Pd based catalyst and boronic acid (Scheme 10). In this study the protein model system employed was a single cysteine mutant of the subtilisin from *Bacillus lentus* (S156C) serine protease.



Scheme 10. Suzuki-Miyaura coupling on a protein substrate

The first report on gold-mediated cysteine modification of peptides came from Chan *et al*..[108] They reported the highly efficient and selective labelling of cysteines in peptides and proteins when using various functionalised allenes in the presence of a mixture of AuCl-AgOTf. The strategy was used to label reduced RNaseA protein, leading to the hydroxyl vinyl thioether protein conjugate **12** (Scheme 11).



Scheme 11. Gold-catalysed cysteine labelling with allenes on a protein substrate

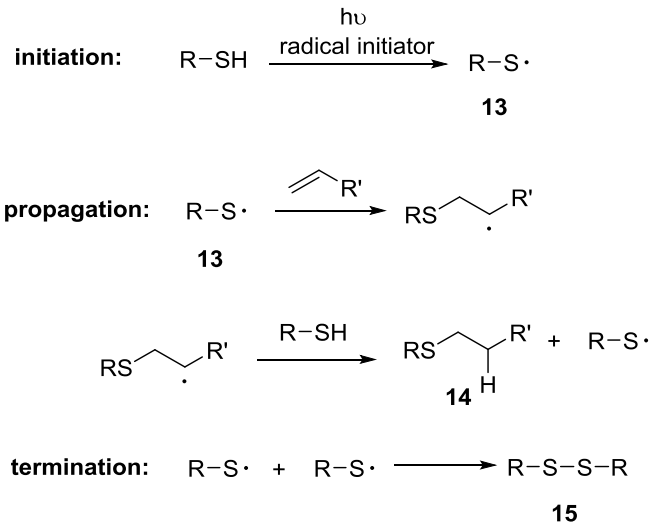
In conclusion, metal-mediated reactions are emerging as very powerful tools for chemical modification of proteins.

I.2.2.6 Photo-induced cysteine labelling

In recent years, the thermally or photochemically induced radical additions of thiols to alkene or alkynes - known as the thiol-ene and the thiol-yne couplings respectively, and first reported in the mid-1900s[109] - have gained significant importance in both polymer science and bioconjugation, as proven by numerous

applications described in the literature.[110–112] A detailed review on the thiol-ene reaction is provided by Bowman and Hoyle,[113] while the Dondoni group has published on the less exploited thiol-yne reaction.[114,115] These reactions are biocompatible due to a series of properties such as efficiency, orthogonality to a wide range of functional groups, compatibility with water and oxygen and no need of metal catalysts. The transformation allows the introduction of various tags and the resulting product is generally stable under a wide range of conditions (acidic and basic, oxidizing and reducing environments).

The reaction mechanism for the thiol-yne reaction is depicted in Scheme 12.[116] The first step is the thiyl radical **13** addition to the alkene to give, after hydrogen abstraction from another thiol molecule, the thioether product **14**. The main side product in this transformation is disulphide **15** arising from thiol homo-coupling. However, this problem can be fixed by using a small thiol such as DTT which will regenerate the thiol.

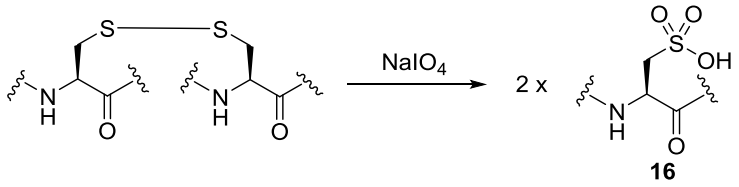


Scheme 12. Mechanism for the thiol-ene reaction

In the area of site-selective modification of protein, the thiol-ene and thiol-yne reactions have been used to mimic post-translational modifications such as acetylation[117] and glycosylation,[115,118] to introduce tags containing lanthanide binding sites for paramagnetic NMR[119] and to form photo-switchable bridges,[120] as a way of controlling conformational domains in biomolecules.

I.2.3 Cystine (disulphide bond) modification

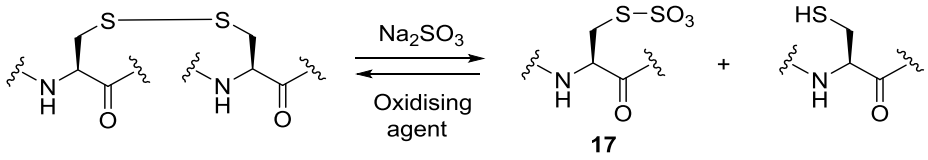
Traditional methods involved cleavage of the cystine by oxidation with sodium periodate in 0.1 M to 1.0 M HCl to give two molecules of cysteic acid **16** (Scheme 13).[121]



16

Scheme 13. Modification of cystine with sodium periodate

An alternative was represented by the use of sodium sulphite which, when reacted with a disulphide bond at pH 8 - 9, gives the *S*-sulfo derivative of cysteine **17** and cysteine (Scheme 14). The addition of an oxidising agent such as cupric ions or *o*-iodosobenzoate ensures that all cysteines are converted to the *S*-sulfo product.[122]

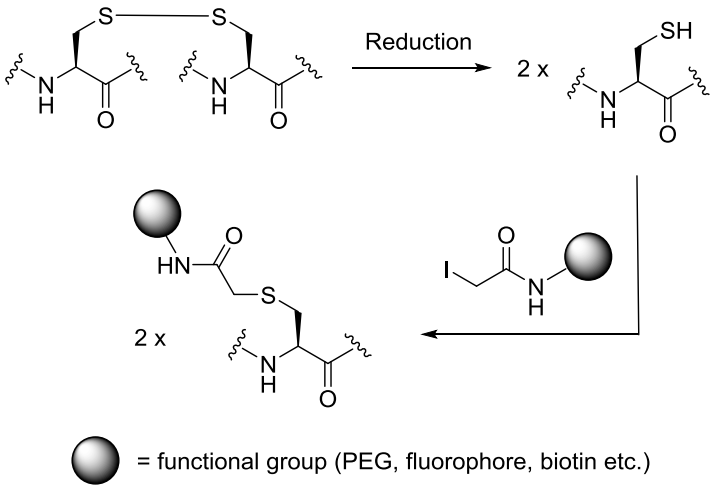


17

Scheme 14. Sulfitolysis of cystine

Despite the fact that these transformations have provided a useful insight into the study of proteins and the roles of disulphide bonds, they are often accompanied by side reactions such as methionine oxidation[123] and tyrosine modification.[124]

More recently,[125] the modification of disulphide residues in proteins is achieved *via* reduction of the disulphide bond followed by alkylation of the resulting thiol groups with alkylating agents (e.g. α-haloacetamides), (Scheme 15).



Scheme 15. Cystine reduction followed by alkylation of cysteine

The reduction step employs thiols such as 2-mercaptoethanol **6**[126] and dithiothreitol **7**[127] (Scheme 4) or trialkylphosphines such as *n*-tributylphosphine **18** [128] and tris(2-carboxyethyl)phosphine **19** [129,130] (TCEP), (Fig. 6). 2-mercaptoethanol, dithiothreitol and TCEP are particularly useful due to their solubility in water.

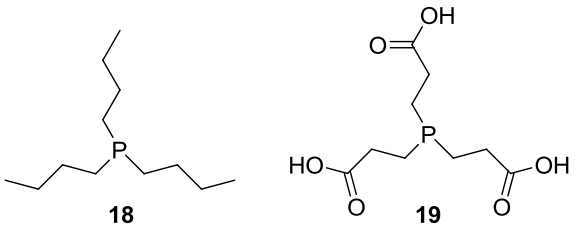
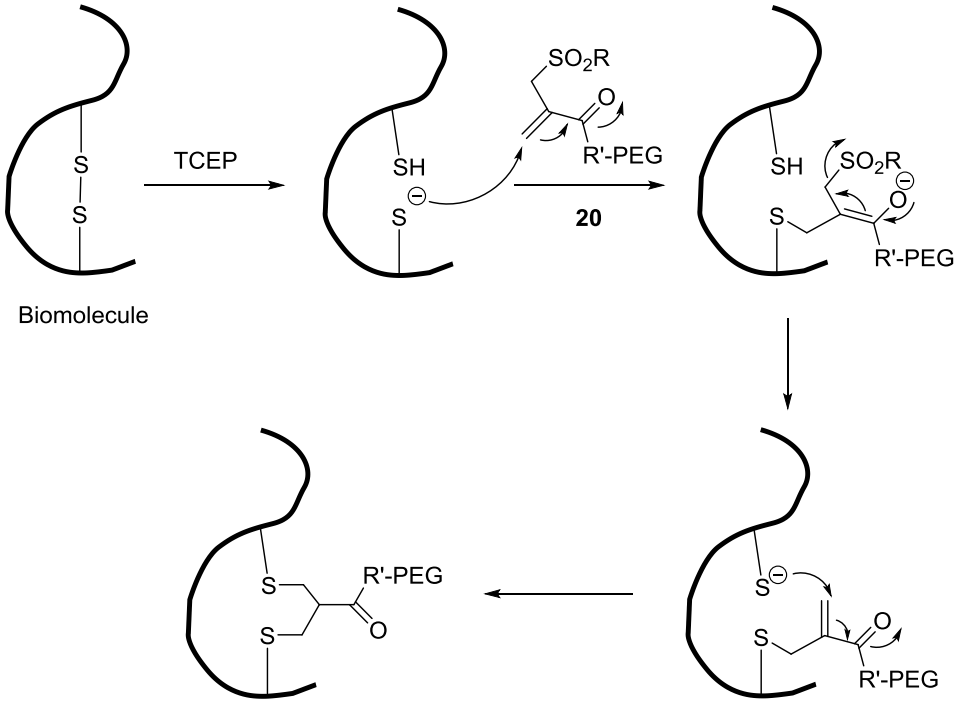


Fig. 6. Commonly employed trialkylphosphine based reducing agents

It is noteworthy to mention that during the reduction step, disulphide scrambling followed by protein aggregation is possible, as the conformation of many proteins depends on the disulphide bonds. Ideally the labelling strategy would introduce the tag very rapidly and retain the bridge. Also, the design of bridging reagents should ensure that the conformational changes induced to the biomolecule by the labelling reagent are kept to a minimum.

Brocchini and co-workers[131–133] developed a strategy that allows functionalisation of disulphides whilst following these principles. Their solution was to use a reagent able to rebridge the two cysteine residues, mimicking the role of the disulphide bond; in this way, it is hoped that the protein's structure and function are unaltered. The bridging reagent **20** consists of an α,β-unsaturated carbonyl compound with a sulfonyl as a leaving group at the *β'* position (Scheme 16).



Scheme 16. Strategy for disulphide bridging with PEG functionalised reagent

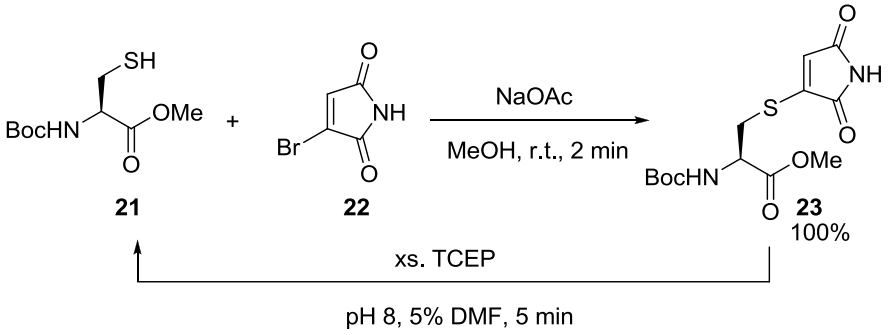
An additional advantage of reagent **20** is its size; the bridge formed between the two thiol groups is just three carbon atoms, which lowers the possibility of inducing a significant change in the protein structure upon its incorporation. Additionally, it can be easily synthesised. Balan *et al.*[132] successfully applied their strategy for the modification of the tripeptide glutathione, the cyclic peptide hormone somatostatin, the tetrameric protein *L*-asparaginase, and to the disulphides in interferon α-2b (IFN). An important limitation of this strategy is the fact that in the time between the reduction of the disulphide bond and the alkylation of the thiols, processes such as disulphide scrambling,[134] aggregation[135,136] and loss of activity[62,137] of the protein could occur.

I.3 Bromomaleimides in protein modification

In 2009, the Baker group reported on bromomaleimides as the first of a new class of reagents that could be efficiently used for the selective and reversible modification of cysteine.[138]

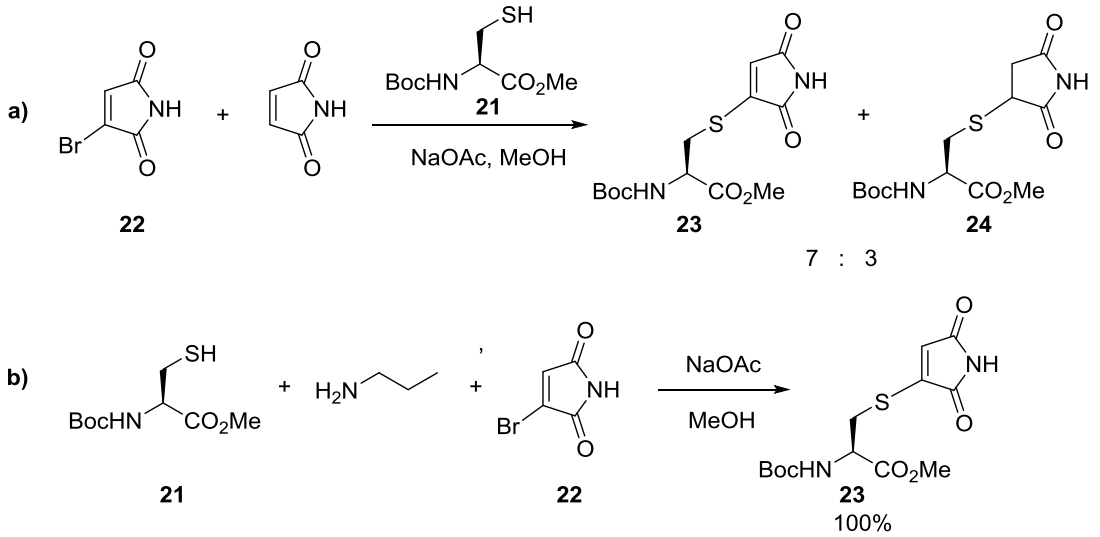
Firstly it was shown that the commercially available *N*-Boc-Cys-OMe **21,** which was used as a model for the amino acid cysteine reacts extremely rapidly (<1 min) and cleanly with bromomaleimide **22**, giving thiomaleimide **23** quantitatively. The reaction is a two-step transformation involving the attack of the thiol on the double bond of the maleimide, followed by elimination of the bromide.

Thiomaleimide **23** is susceptible to further nucleophilic attack due to the double bond in the ring. The same paper describes how this feature was used to demonstrate the ease of reversing the reaction, by using excess TCEP **19**, the commonly used water soluble phosphine (Scheme 17).



Scheme 17. Bromomaleimide **22** addition to cysteine **21**, followed by TCEP induced cleavage of the resulting thiomaleimide **23**

Initial competition experiments between bromomaleimide **22** and maleimide showed that the former is faster in reacting with the protected cysteine **21** (Scheme 18a). In another competition experiment, the protected cysteine **21**, in the presence of equimolar quantities of propylamine and bromomaleimide **22**, was shown to react solely with the latter (Scheme 18b) - suggesting the potential use of this reagent to selectively label cysteine over lysine in the context of a biological system.

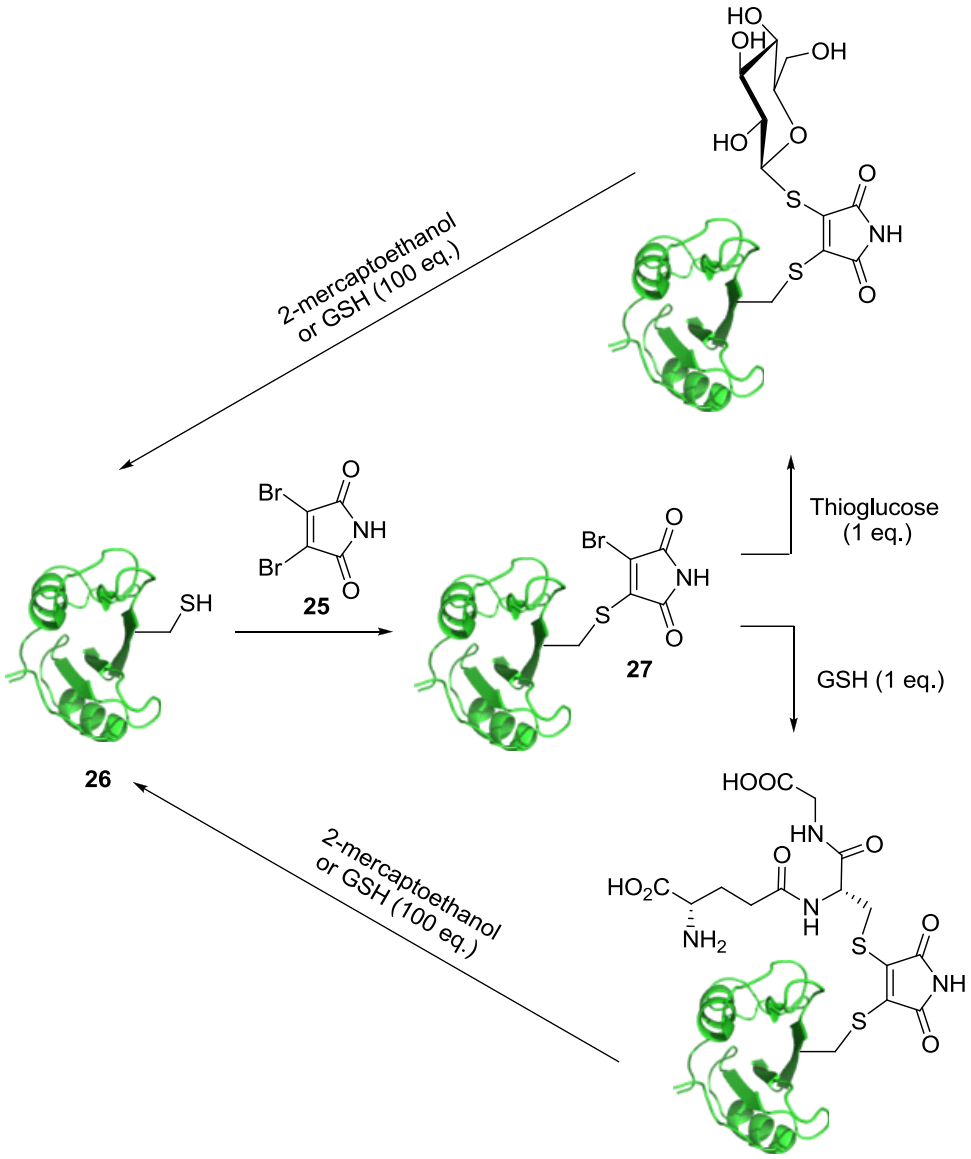


Scheme 18. Competition experiments involving bromomaleimides

Further studies into the utility of bromomaleimides in biological systems were carried out in collaboration with the groups of Professor Caddick (UCL, Chemistry)

and of Professor Waksman (Birkbeck, ISMB). The most relevant results are highlighted in the following paragraphs.

Scheme 19 illustrates how dibromomaleimide **25** was used to label the Grb2 SH2 (L111C) adaptor protein **26** and how product **27** was further functionalised with thioglucose and glutathione respectively.[139]

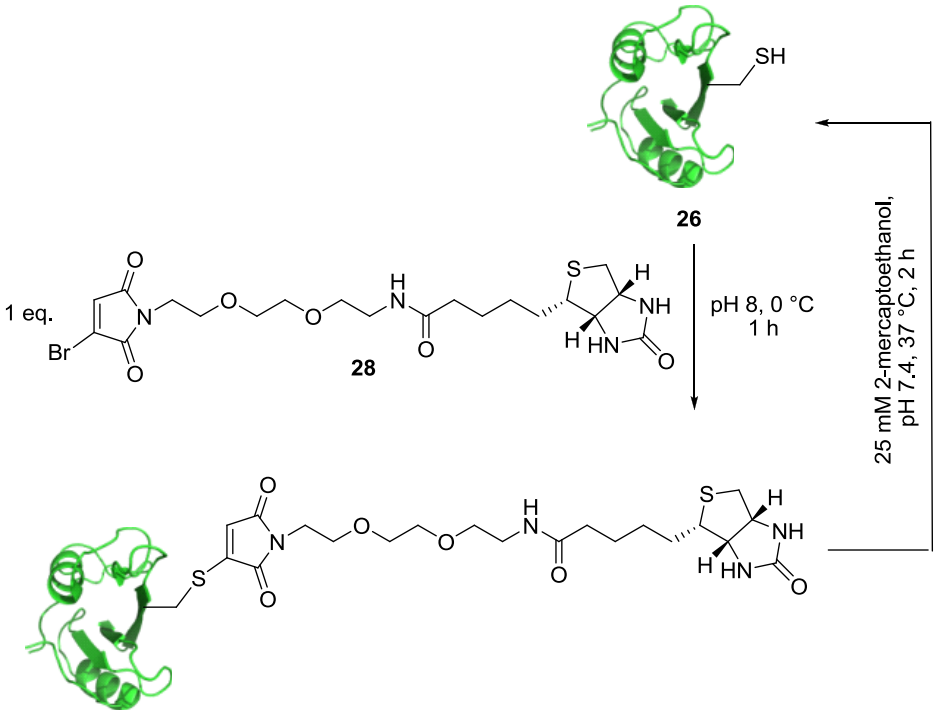


Scheme 19. Reversible modification of Grb2 SH2 (L111C) **26** (PDB id: 3MXC) with dibromomaleimide **25** followed by further functionalisation with thioglucose or GSH

Reactions were fast and reached full conversion within minutes and required as little as 1 eq. of the labelling reagent. The cleavage of the protein conjugates was also performed. Cleavage to afford the initial protein proved facile when using excess (100 eq.) of glutathione or 2-mercaptoethanol. This shows that

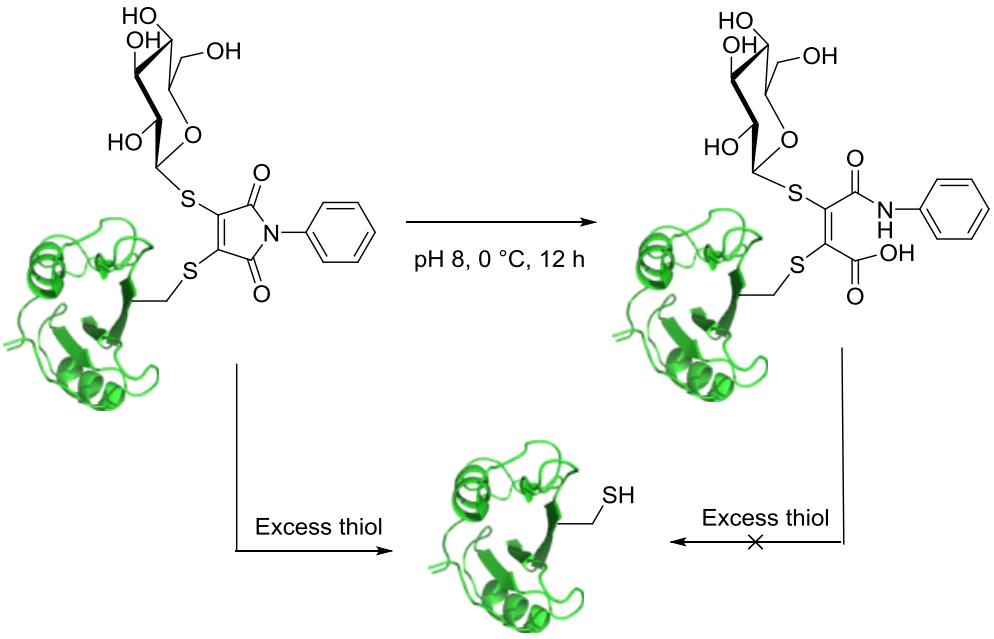
thiomaleimides are susceptible to thiol exchange in the presence of excess thiol and establishes the addition of excess thiol as a useful method to reverse the transformation.

A direct application of bromomaleimides in the temporary labelling of protein is in the biotin-labelled protein enrichment. The biotin-avidin interaction is a well established technique for protein purification, however it suffers from the limitation that harsh conditions are required to release the protein (e.g. high concentration of denaturant, high temperatures and very low pH) after immobilisation.[140] It was shown by Ryan[141] *et al.,* by using bromomaleimide **28** that biotinylation using a thiomaleimide linker allows cleavage under mild conditions of the immobilised protein, affording the native protein **26** (Scheme 20).



Scheme 20. Reversible biotinylation of Grb2 SH2 (L111C) with bromomaleimide **28**

Whilst the utility of bromomaleimides as reversible cysteine labelling reagents is a major asset, there are cases where a permanent, irreversible transformation is desired. The Baker and Caddick groups reported on the controlled hydrolysis of the *N*-aryl maleimides leading to the corresponding maleamic acids that are no longer cleavable in thiol rich media.[141] The hydrolysis protocol that was established is an elegant way to render the attachment of maleimide irreversible. The concept was demonstrated on Grb2 SH2 (L111C), (Scheme 21).

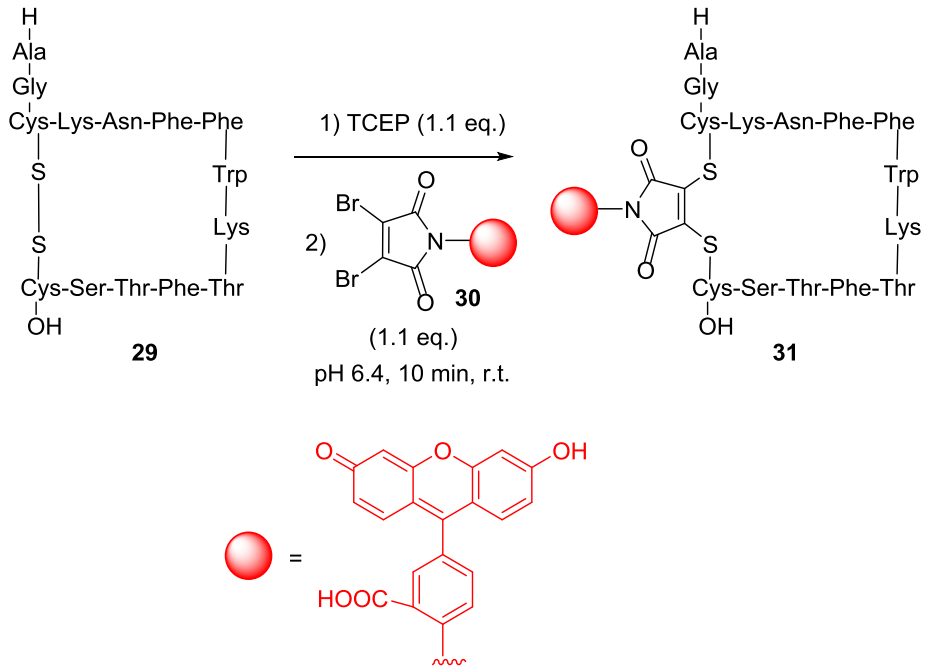


Scheme 21. Thiol cleavable and thiol non-cleavable Grb2 SH2 (L111C) conjugates

The use of dibromomaleimides for the modification of disulphide bonds was also investigated. Similarly to the work of Brocchini and co-workers, the Baker group tested the ability of dibromomaleimides to act as bridging reagents that could insert into the disulphide bonds of proteins, while retaining the structure and function of the macromolecule.[142]

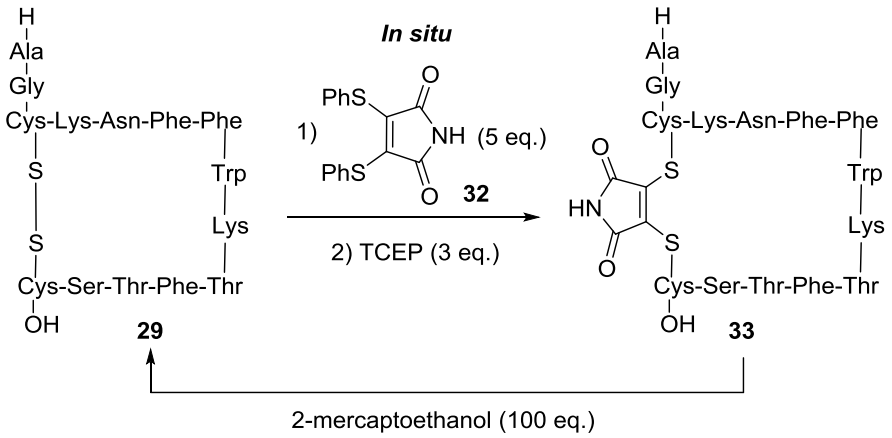
Indeed, various functionalised dibromomaleimides were incorporated in the single disulphide of somatostatin **29**. Somatostatin is a 14-amino acid cyclic peptide with widespread effects on brain, pancreas, gut and central nervous system functions. It inhibits the release of the growth hormone as well as insulin, rennin, acetylcholine, calcitonin and various other hormones[143]. Octreotide and lanreotide, analogues of somatostatin, are being used in the treatment of acromegaly.[144] Somatostatin **29** contains a single disulphide bridge between Cys3 and Cys14, essential for maintaining the active conformation, which provided a suitable model system for testing the reactivity of disulphides towards double bonds.[145]

The reaction was shown to work well when using *N*-fluorescein dibromomaleimide **30**, affording the fluorescent bridged somatostatin conjugate **31** in quantitative conversion after 10 min (Scheme 22). The process consisted of two steps: 1) reduction of the disulphide with TCEP **19** (1.1 eq.) and 2) addition of dibromomaleimide **30** (1.1 eq.).



Scheme 22. Stepwise bridging of disulphide bond in somatostatin with *N*-fluorescein dibromomaleimide **30**

Even though the bridging rate is high, the strategy suffers from the same limitations as other established methodologies for disulphide bridging: risk of protein aggregation, disulphide scrambling and subsequent loss of activity in the time that passes from the moment the bond is reduced and the moment it is fully bridged. To eliminate the risk of disulphide scrambling, it would be beneficial to trap the cysteine groups, released after treatment of the disulphide with the reducing agent with the bridging reagent as soon as possible. So ideally, the reduction and bridging steps would take place in tandem, which will henceforth be referred to as an "*in situ*" process. This involves mixing the disulphide bond containing biomolecule, the reducing and the bridging reagents altogether: the bridging would occur as soon as the disulphide is reduced. A crucial condition for the development of such an *in situ* process is that the bridging reagent does not show any cross-reactivity with the reducing agent. Dibromomaleimides proved not to satisfy this condition, undergoing TCEP attack. To solve this problem, dithiophenolmaleimide **32** was developed. The new protocol consists in the addition of TCEP (3 eq.) to a pre-mixed solution of somatostatin **29** and dithiophenolmaleimide **32** (5 eq.). Under these conditions, the reaction was complete in less than 20 min, affording bridged somatostatin **33**. Furthermore, the reaction could be reversed by adding excess 2-mercaptoethnaol (100 eq.), (Scheme 23).

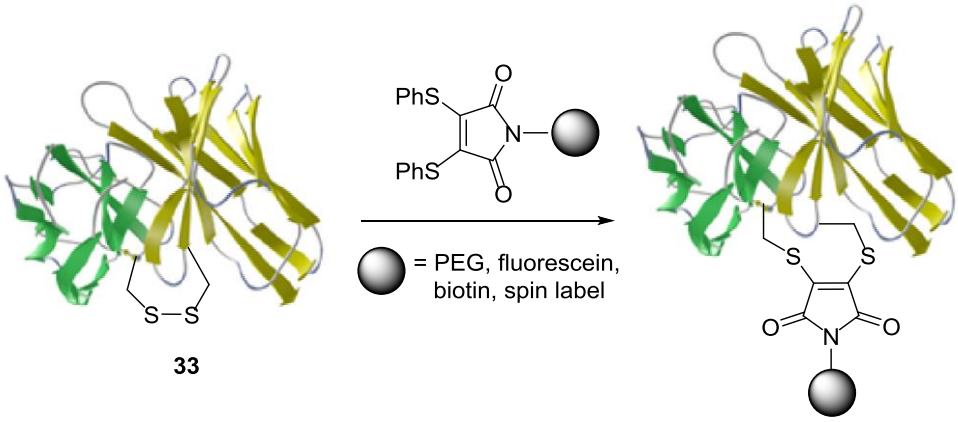


Scheme 23. *In situ* somatostatin bridging with dithiomaleimide **32**

As mentioned before, one risk of choosing disulphide labelling as a bioconjugation strategy is that by perturbing the disulphide bond, the biomolecule loses its biological activity. To test whether the maleimide bridge insertion affected the activity of the peptide, the synthesised maleimide bridged analogues were subjected to patch-clamping. The result was favourable, showing that the bioconjugates still maintained the reported activity in the activation of GIRK channels.[142]

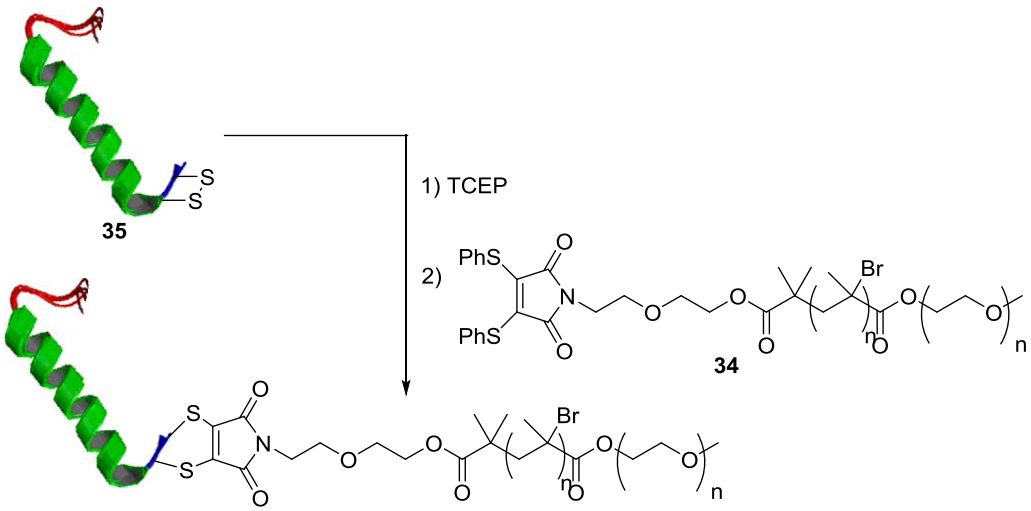
Some essential advantages of using disubstituted maleimides over the reagents used by Brocchini and co-workers are the fact that no asymmetric centre is introduced into the system and that the bridge between the two sulphur atoms is of just two carbons connected by a double bond; hence, the conformational changes in the protein are expected to be minimal.

The Baker and Caddick groups have also shown that the bridging with *N*-functionalised dithiophenolmaleimides is also efficient on larger substrates such as scFv antibody fragments (Scheme 24).[146] They have shown that the bridged products retain full binding activity and display good plasma stability. The successful antigen detection by spin labelled ds-scFv **33** was achieved by continuous-wave electron paramagnetic resonance (cw-EPR) which highlights the potential of using this bridging strategy for diagnosis.



Scheme 24. Bridging of ds-scFv **33** with functionalised dithiophenomaleimides (representation of the protein was taken from Schumacher *et. al.*)[146]

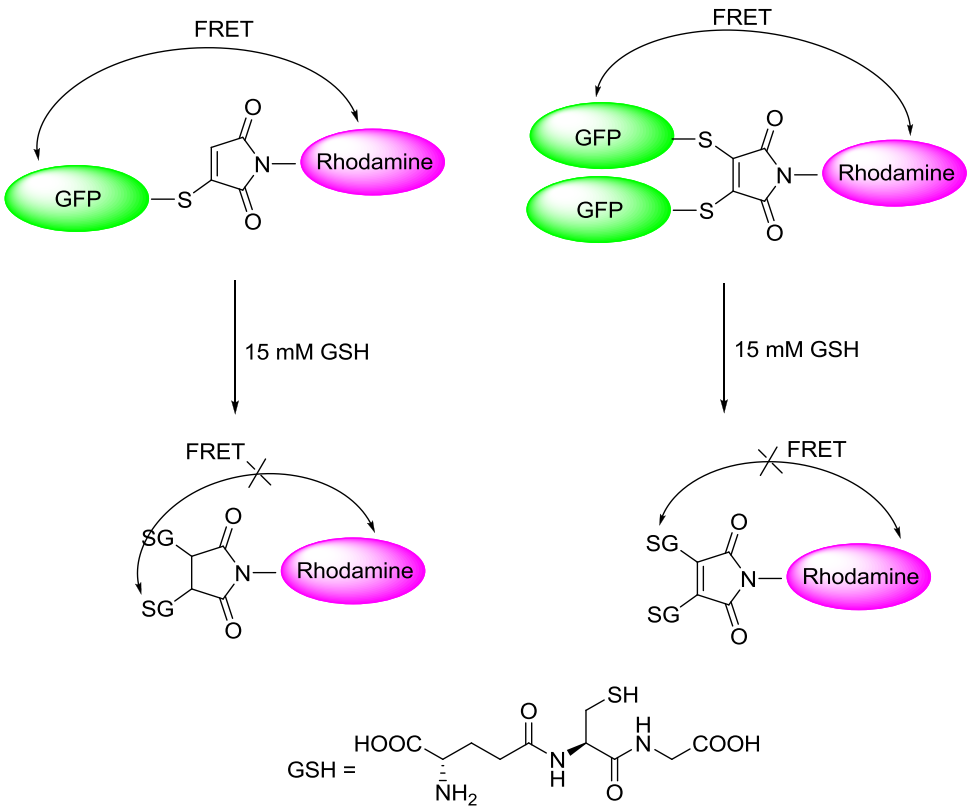
Jones *et al.*[147] have successfully used dithiophenolmaleimides in atom transfer radical polymerisation. In 2011 they reported on the first example of an unprotected maleimide-functionalised initiator tolerant to polymerisation conditions and used the resulting dithiophenol maleimide functionalised polymers such as **34** to bridge the disulphide bond in the 32-amino acid peptide hormone calcitonin **35** (Scheme 25). Dibromomaleimide-based functional ATRP initiators were also tested but these led to polymerisation inhibition and therefore the dibromomaleimide moiety, essential for the disulphide bridging, was added to the polymer at a later stage.[148]



Scheme 25. Disulphide bridging of sCT with polymer *via* maleimide linker (Jones *et al.*)[147]

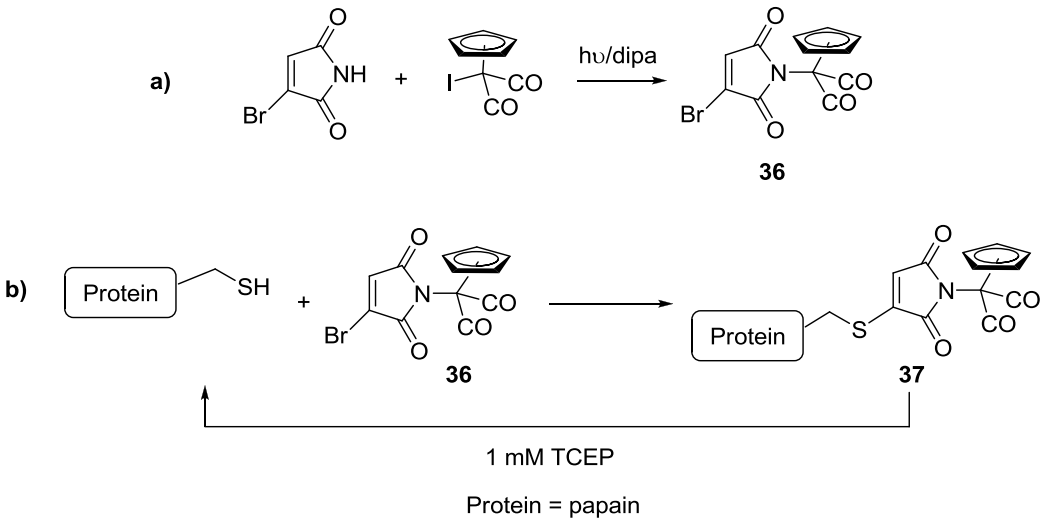
The potential of substituted maleimides as drug carriers was also explored. Using a series of bromomaleimide-GFP (green fluorescent protein) conjugates

(Scheme 26), designed as FRET pairs, the release of the protein in the mammalian cells was demonstrated (by microinjection).[149] The cleavage of the bioconjugates is based on the reaction with the excess glutathione present in the cytoplasm and on the ability on thiomaleimides to undergo thiol exchange.



Scheme 26. Cleavage of GFP-rhodamine conjugates with maleimide linker in the presence of GSH

Another area where bromomaleimides have proven useful was in the synthesis of metallocarbonyl complex-based protein biomarkers and also enzyme inhibitors, as shown by Rudolf *et al*..[150] These applications are based on the fact that maleimidato metallocarbonyl complexes display a strong IR absorbance between 1950 - 2060 cm^{-1}, where biomolecules do not absorb and on their ability to covalently attach to cysteine groups in enzymes. It was shown that the metallocarbonyl bromomaleimide complex **36** reacts efficiently with the single cysteine in papain, inhibiting its activity of the enzyme. Furthermore, the resulting papain thiomaleimide conjugate **37** can be cleaved by addition of TCEP, regenerating the catalytically active enzyme (Scheme 27).



Scheme 27. (a) Synthesis of maleimidato metallocarbonyl complex **36** and (b) Reaction between **36** and the cysteine in papain

All these examples show that bromomaleimides are very versatile trifunctional scaffolds with various applications in the area of site-selective protein modification: temporary blockage of an active cysteine residue, protein purification, quantitative and qualitative analysis of proteins, probing binding sites, synthesis of bioconjugates and drug delivery.

I.4 Methods for the dual modification of proteins

The ability to introduce homogenously and site-specifically two different functionalities onto a protein can give rise to new potential application. Examples include pegylation of a protein-drug conjugate to increase its *in vivo* half-life or fluorescent tagging of protein-drug conjugates for visualisation studies. Additionally, the incorporation of two fluorophores that form a FRET pair, enables FRET based structural studies of proteins.

Solid phase synthesis facilitates differential labelling of peptides, simply by incorporating already labelled amino acids or non-natural amino acids which will allow the subsequent labelling with the desired tag.[151] So far, several approaches - based both on chemical and molecular biology methods - have been developed for the differential modification of proteins. The most representative methodologies will be reported, as well as some of their applications.

A general feature of the methods currently available is that they require either the introduction of one or more amino acids at a chosen location (this can be

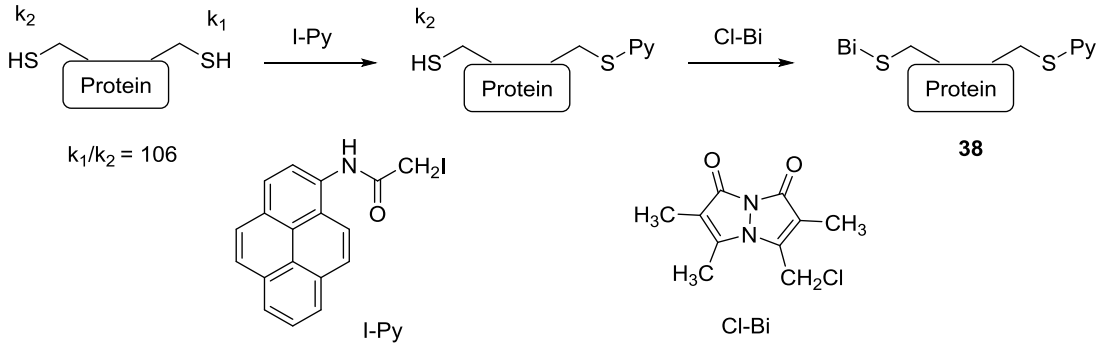
done by site-directed mutagenesis) or of a non-natural amino acid (by the modification of the cellular ribosomal machinery).

Each extra step (with or without purification) involved in the preparation of the dual labelled biomolecules contributes to the decrease of the overall yield and makes the method more tedious and time-consuming. While for some applications such as FRET, sub-milligram quantities of labelled protein are necessary, for stop-flow experiments used to investigate reaction kinetics, much larger amounts of protein are required. In this context, simpler and more general methodologies that are suitable for scale-up are needed.

The major hurdle when sequential covalent modification of two different sites of a protein is attempted is orthogonality. Ideally each of the two labelling reagents would exclusively react with its target. In the absence of this selectivity, heterogeneous product mixtures are formed, which are associated with tedious and generally very low-yielding purification steps. Currently, there are several strategies for selectively modifying one amino acid in the presence of others: (a) targeting two natural amino acids that display very different reactivities,[152–154] (b) targeting a natural and a non-natural amino acid[155,156] or (c) targeting two orthogonal non-natural amino acids.[157,158] A separate set of dual modification strategies are based on the incorporation in the biomolecule of a dual-labelled moiety that contains two orthogonal groups (e.g. azide and alkyne, azide and ketone) which can then be labelled with the two tags that are required for the application.[159,160] As with all protein engineering techniques, care should be taken that the modifications made do not induce any significant conformational changes or loss of activity of the native protein. This is achieved by designing mutations that are as conservative as possible and that do not interfere with the residues known to be involved in the protein function.

A representative example of dual labelling based on two natural amino acids that display different reactivities is detailed in a study by Ratner *et al.*.[161] Using *Escherichia coli* adenylate kinase (AKe) as a model protein, they have generated a series of dual cysteine mutants for which they have calculated the rate constants specific to each of the cysteines when reacted with a reference reagent (Ellman's reagent). When designing mutants, the 3-D structure of the native protein is a very useful tool as several factors that are known to affect the reactivity of the cysteine can be estimated in this way. Such factors include solvent accessibility, neighbouring group effects and hydrogen bonding. From all the mutant candidates,

AKe (V169C/A188C) was selected for having the highest ratio (106) between the rate constants of two cysteine groups. This was then sequentially labelled with a donor - (I-Py) [*N*-(1-pyrenylmethyl)iodoacetamide] - and acceptor - (Cl-Bi [monochlorobimane] - fluorophore molecule respectively, known to form a FRET pair (Scheme 28).

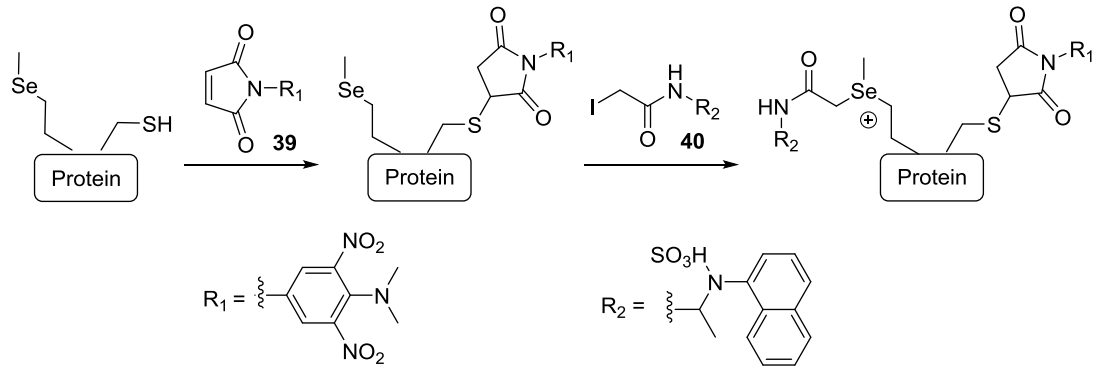


Protein = *Escherichia coli* adenylate kinase (V169C/A188C)

Scheme 28. Dual labelling strategy of a dual cysteine mutant of AKe

In the end, the doubly labelled protein **38** was purified by hydrophobic interaction chromatography. The protocol was efficient as overall yields ranged between 70% and 90% affording tens of milligrams of product. Additionally, the dual modified AKe mutant retained full enzymatic activity and reversible folding-unfolding transition ability. FRET based analysis of the dual modified AKe allowed the team to calculate distances between the two modified residues upon folding and unfolding and therefore get more insight into its conformation.

Another pair of naturally occurring amino acids that can be easily incorporated and that show a significant difference in reactivity is cysteine and selenomethionine. Selenomethionine was shown to react with electrophiles such as iodoacetamides[154] and benzyl bromide[162] derivatives but not with maleimides. Therefore it was envisaged that a pair or cysteine and selenomethionine can be selectively labelled, without cross-reactivity. The efficiency of the strategy was shown on the double mutants of the streptococcal protein CAMP factor and calmodulin protein, each containing a selenomethionine and cysteine residue respectively: first the cysteine was labelled with a functionalised maleimide **39** and then the selenomethionine was labelled with a functionalised iodoacetamide **40** (Scheme 29).[154]

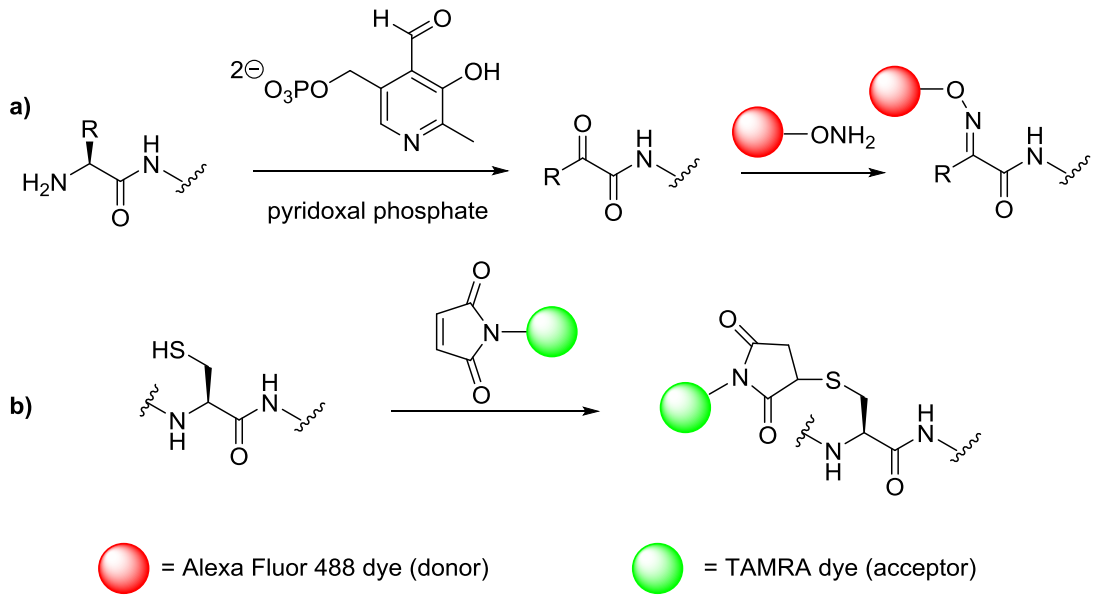


Protein = 13SeM-137C-CAMP-L2 and 34SeM-110C-CaM-L9

Scheme 29. Dual labelling strategy shown on double mutants of the CAMP factor and calmodulin (CaM) proteins

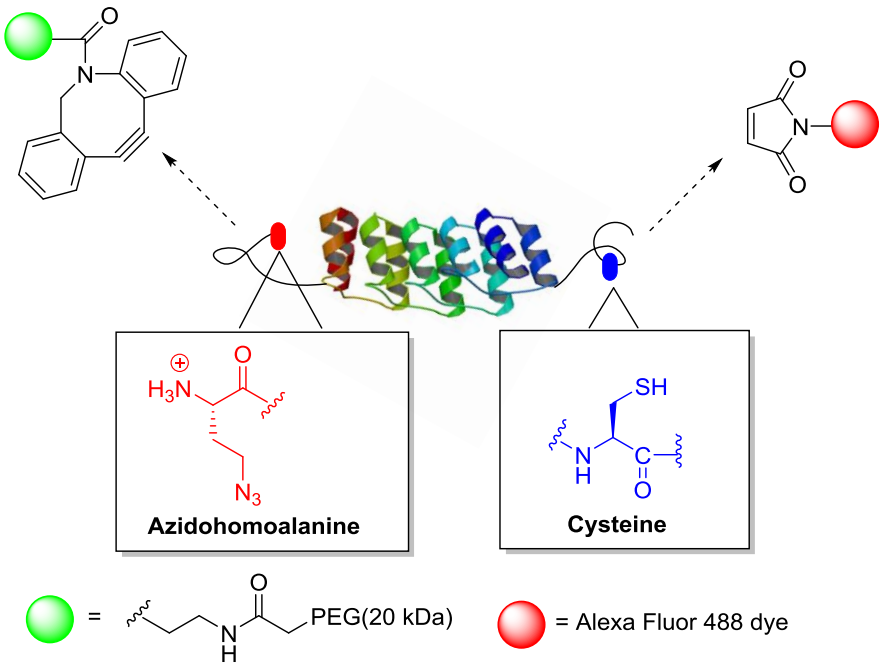
A drawback of this strategy is the stability of selenomethionine, which in certain situations, especially when in close proximity to carboxylic acid side chains, can undergo side reactions.

Chrochet *et al.*'s[163] strategy is based on the orthogonality between cysteine labelling with maleimides and the transamination reaction involving amino-terminal group. The transamination reaction is mediated by pyridoxal 5'-phosphate and results in the installation of a ketone or aldehyde at the *N*-terminus of the protein, which can then be reacted chemoselectively with alkoxyamine-based reagents *via* oxime formation. Using this strategy, they have dual labelled mutants of single cysteine mutants of the periplasmic binding protein (PBP) with two commercially available fluorophores (Alexa Fluor 488 and TAMRA) which then can act as biosensors for monitoring the protein binding to specific ligands (Scheme 30). The limitation of this method is that, while the cysteine can be inserted in various positions, the second label is restricted to the *N*-terminus of the protein.



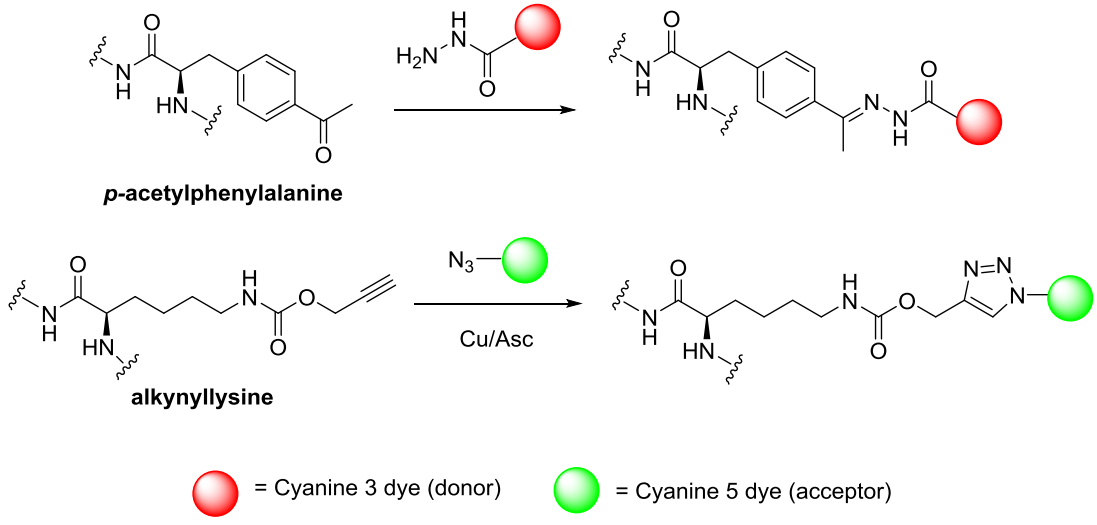
Scheme 30. Chemoselective labelling of a) the *N*-terminus and b) of the inserted cysteine (Crochet *et al.)*[163]

The combination of thiol and click chemistry makes the approach chosen by the Pluckthun[155] group for the dual functionalisation of the Designed Ankyrin Repeat Proteins (DARPins) very efficient and versatile. DARPins are a new class of therapeutic proteins that can be used as an alternative to antibodies.[164] They can be expressed as to contain clickable methionine surrogates such as azidohomoalanine (Aha) or homopropargylglycine (Hpg) at the *N*-terminus, whilst retaining their native activity. This is done after ensuring that the only codon for methionine is the one at the *N*-terminus by performing the expression in a methionine auxotrophic medium to which the clickable methionine surrogate had been previously added. In addition, they are also devoid of cysteines therefore a single cysteine can be inserted *via* site-directed mutagenesis allowing single attachment of cysteine specific reagents. The group exploited these two features and synthesised several examples of Aha or Hpg and single cysteine containing DARPin mutants which they then functionalised with a PEG chain *via* a strain-promoted azido - alkyne cycloaddition and with a fluorophore *via* maleimide conjugate addition (Scheme 31). The dual labelled DARPins were used to measure binding kinetics on tumour cells.



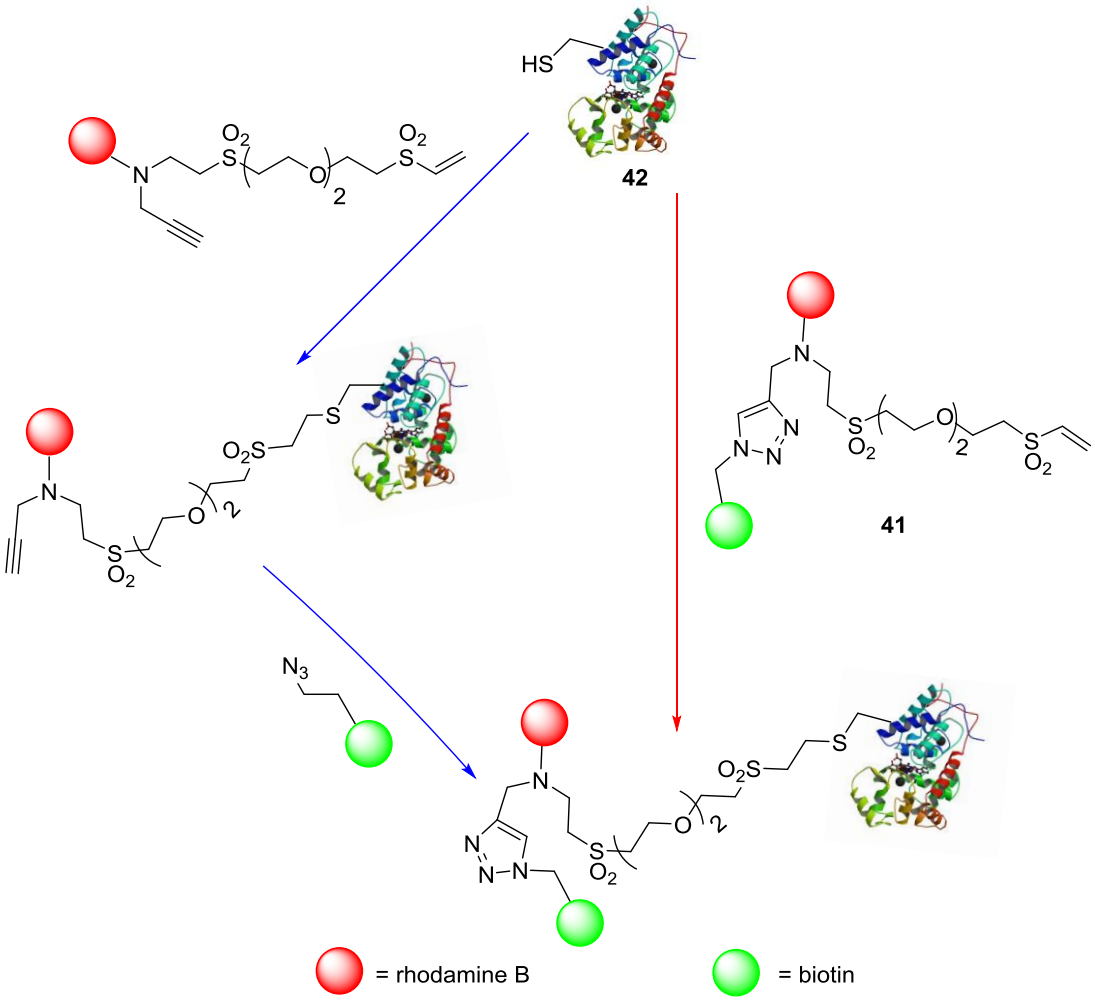
Scheme 31. Strategy for the chemoselective dual functionalisation of DARPins (Simon *et al.*)[155]

Another approach involving the incorporation of two non-natural amino acids used for the dual functionalisation of calmodulin, which was then used to probe the conformational changes of this protein upon binding to Ca^{2+} and the M13 peptide.[157] The two unnatural amino acids introduced were *p*-acetylphenylalanine and alkynyllysine which were then labelled with two fluorophores that form a FRET pair (Cyanine 3 and Cyanine 5) using a hydrazine reaction and a Cu-catalysed azido-alkyne cycloaddition reaction respectively (Scheme 32).



Scheme 32. Dual labelling strategy based on the modification of two unnatural amino acids (Park *et al.*)[157]

If the two labels are not required to be on different amino acids, then the easiest approach is to label the biomolecule with a multifunctional tag that either contains the two labels desired or can be subsequently chemoselectively modified with the desired labels. A very useful such scaffold is represented by the vinyl sulfone bifunctional tag reagent **41** (Scheme 33) developed by Santoyo-Gonzales *et al*. that are designed to target cysteines.[159] In this paper the two tags on the bifunctional reagent **41** were a fluorophore (rhodamine B) and biotin while the model system employed was Horse Radish Peroxidase **42**.

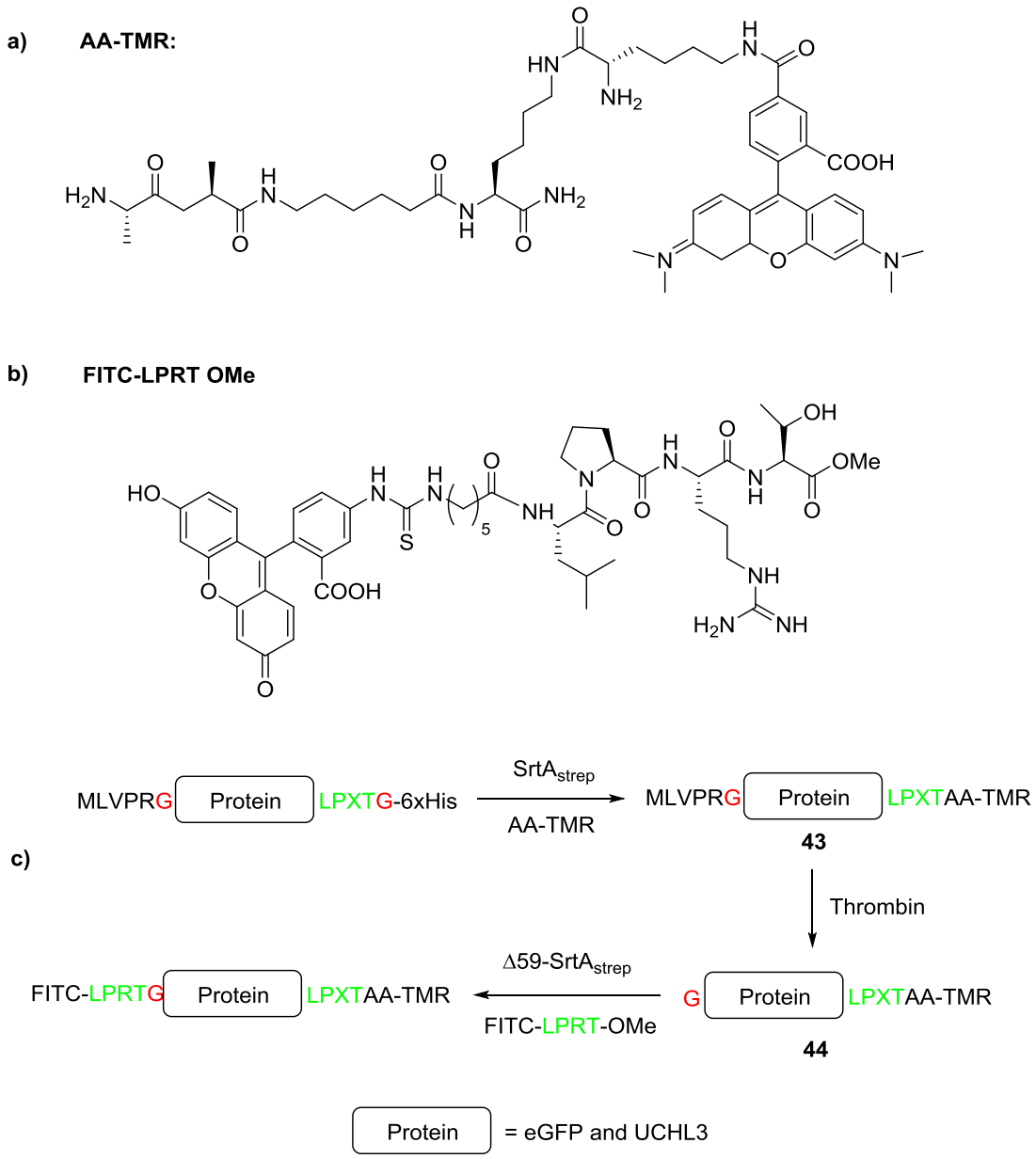


Scheme 33. One-step (red arrow) *vs.* two-step strategy (blue arrows) for the dual functionalisation of Horse Radish Peroxidase (Santoyo-Gonzales *et al*.)[159]

Enzymatic approaches for the dual modification of proteins have gained significant importance.[165,166] These methods use sortases that catalyse transpeptidation reactions. Target proteins are engineered to have, at both ends, the recognition site necessary for the enzyme. In the presence of a labelled peptide, the

transpeptidation reaction will take place, thus incorporating the label into the protein. However, this approach is limited to the labelling of the terminal protein region.

A representative study is provided by Antos *et al.*[167] who describe the site-specific *N*- and *C*- terminal labelling of two different proteins (eGFP and UCHL3) using sortases of different selectivities: sortase A from *Staphylococcus aureus* ($SrtA_{staph}$) and the truncated form of sortase A, Δ59-$StrA_{staph}$.The two model proteins contain a LPXTG-His6x motif at the *C*-terminus which is recognized by the sortase A and at the other end a MLVPRG amino acid sequence, which acts as a protecting group for the *N*-terminus of the glycine (Scheme 34). The dual functionalisation protocol starts with the labelling of the protein *C*-terminus by the peptide AA-TMR which contains the first tag, rhodamine B. At this stage purification of **43** is required: the His6x-tagged $SrtA_{staph}$ is removed by Ni-affinity column and the unreacted peptide by using a desalting column. Next, treatment with thrombin results in the deprotection of the *N*-terminal glycine residue. The resulting *N*-terminus of the **44** is then coupled to the second peptide which contains the recognition motif needed for the attachment of Δ59-$StrA_{staph}$ and, at the other end, the second tag, fluorescein.

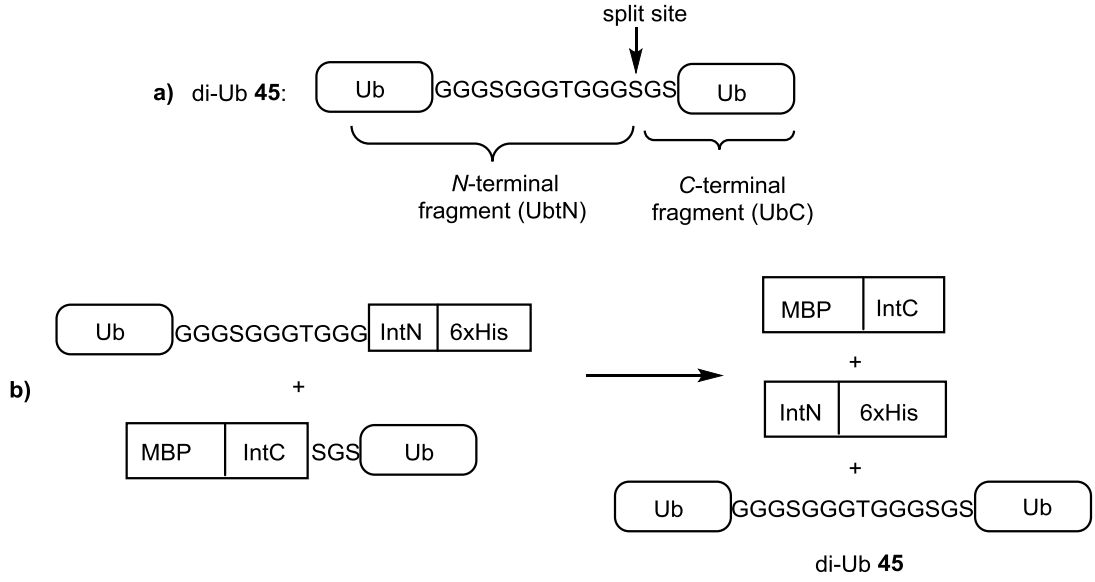


Scheme 34. (a) Tetramethylrhodamine-labelled peptide for $SrtA_{strept}$-mediated modification. (b) Fluorescein labelled peptide for Δ59-$SrtA_{strept}$-mediated modification (c) Strategy for the dual functionalisation of a protein using enzymatic approach (Ploegh *et al.)*[167]

Other approaches for the dual modification of proteins are the expressed protein ligation (EPL) and protein trans-splicing (PTS) protein-ligation methods. They are protein semi-synthesis approaches that allow the synthesis of the target protein from smaller polypeptidic fragments. Both rely on the use of inteins, a class of peptides that undergo self-cleavage when inserted into the precursor protein.[168]

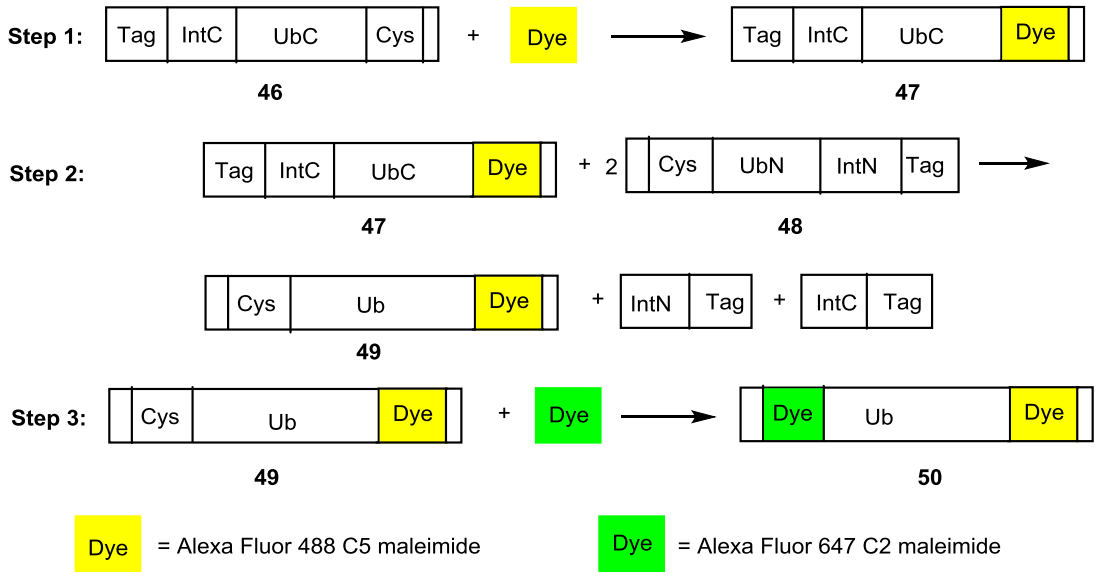
A site-specific two-color protein labelling strategy that utilizes PTS is described by Yang *et al.*.[169] The model protein is an Ubiquitin dimer **45**, with the two

Ubiquitin domains joined by a 14-amino acid linker rich in glycine residues. Two mutations have been performed to allow the site-selective labelling of each of the two fragments: the 47th amino acid (originally a glycine) on the *N*-terminal ubiquitin and the 7th amino acid (originally threonine) on the *C*-terminal ubiquitin were mutated into a cysteine (Scheme 35a). The protein was then divided at the second serine within the linker and expressed as the two fragments: Ub-IntN-6xHis and MBP-IntC-Ub, where MBP is the maltose binding protein, necessary for the stabilization of the protein and 6xHis is a histidine tag that will allow facile purification (Scheme 35b).



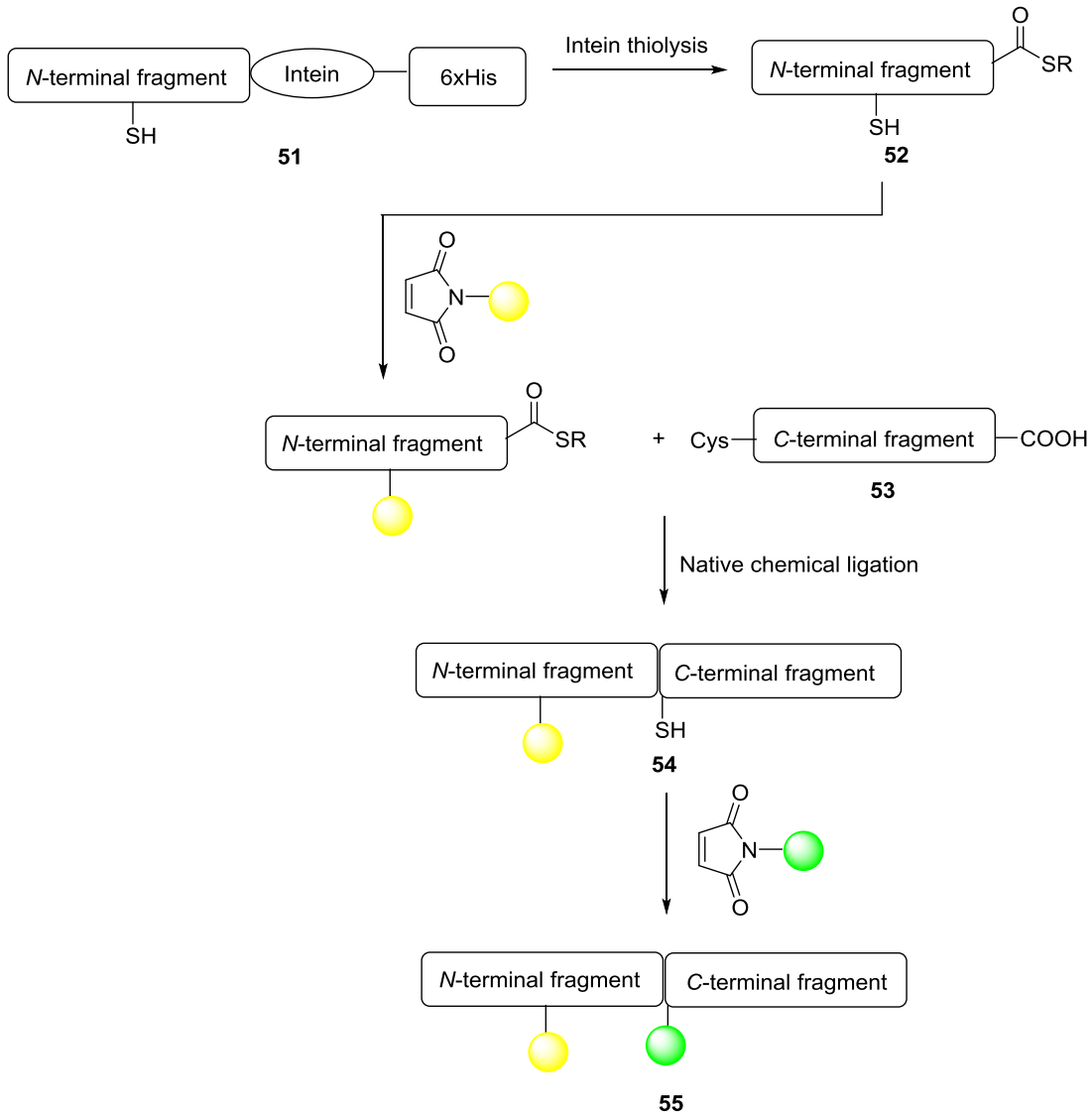
Scheme 35. (a) Di-ubiquitin dimer **45** linked by a peptide linker; (b) Protein trans-splicing between the two intein fused fragments (Yang *et al.)*[169]

The first labelling step takes place at the cysteine on the MBP-IntC-Ub **46** and is achieved by addition of a maleimide fluorophore (Scheme 36). Upon mixing of Ub-IntN-6xHis **48** with the newly labelled MBP-IntC-Ub **46**, the two intein fragments undergo folding to give the MBP-IntC and IntN6xHis (held together by non-covalent interactions) and the labelled di-Ub **49**. Finally the second cysteine in the di-Ub **49** is labelled using a second maleimide fluorophore giving the dual labelled protein, **50**.



Scheme 36. Trans-splicing strategy for the dual labelling of di-Ubiquitin (Yang *et al.)*[169]

EPL has proven useful for the dual labelling of the repeat protein CTPR3 (Consensus TetatricoPeptide Repeat) with a fluorescent pair, as reported by D'Andrea *et al*..[170] EPL consists of the ligation of a *C*-terminal protein containing an *N*-terminal cysteine residue (either obtained by solid phase synthesis or recombinantly) with the thioester *N*-terminal fragment. To allow dual labelling, each fragment is mutated as to be easily singly labelled with the desired tag. In the study of D'Andrea *et al*., the *N*-terminal cysteine residue in **51** is expressed as a fusion protein with the MxeGyrA intein and has a 6xHis tag for purification purposes (Scheme 37). Construct **51** undergoes partial splicing and is then subjected to excess thiol to release the intein moiety and form the thioester *N*-terminal fragment **52**. This is labelled with a maleimide fluorophore and then coupled *via* native chemical ligation with the *C*-terminal protein fragment **53**, containing a *N*-terminal cysteine. Once the two protein fragments are joined together, the second labelling with another maleimide fluorophore is achieved, giving the dual labelled protein **55**.



Scheme 37. Expressed protein ligation strategy for the dual labelling of CTPR3 protein (D'Andrea *et al*.)[170]

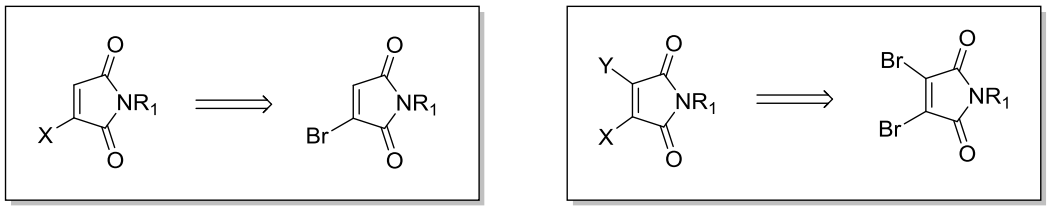
Both EPL and protein *trans*-splicing are powerful methods that have allowed studies in areas such as electrophysiology for the monitoring of membrane proteins such as ion channels,[171] NMR by the introduction of specific isotopes[172] and optical spectrscopy[173,174] all with the aim of probing protein structure and function. An additional strength of these ligation methods is that they can be applied to the synthesis and modification of cyclic polypeptides such as cyclotides.[175]

I.5 Purpose of this work

Continuing the work that has been done in the Baker group, the current project addresses the problem of achieving higher selectivity for cysteine and cystine site-selective modification. Aiming to prove that the efficient cysteine labelling and disulphide bridging reported in the previous sections are not restricted to bromomaleimides, the scope of the substituent(s) on the double bond of the maleimide will be explored.

A library of novel substituted maleimide reagents will be developed that will allow us to determine the influence of the substituent on the reactivity of the compound. Our hypothesis is that by controlling the chemistry of this class of compound, we will be able to tune properties such as thiol selectivity, reactivity, solubility and cross-reactivity with reducing agents and consequently make use of substituted maleimides more efficiently.

The starting point for the creation of this library will be the mono- and dibromomaleimides respectively. Some of our targeted compounds are shown in Fig. 7.



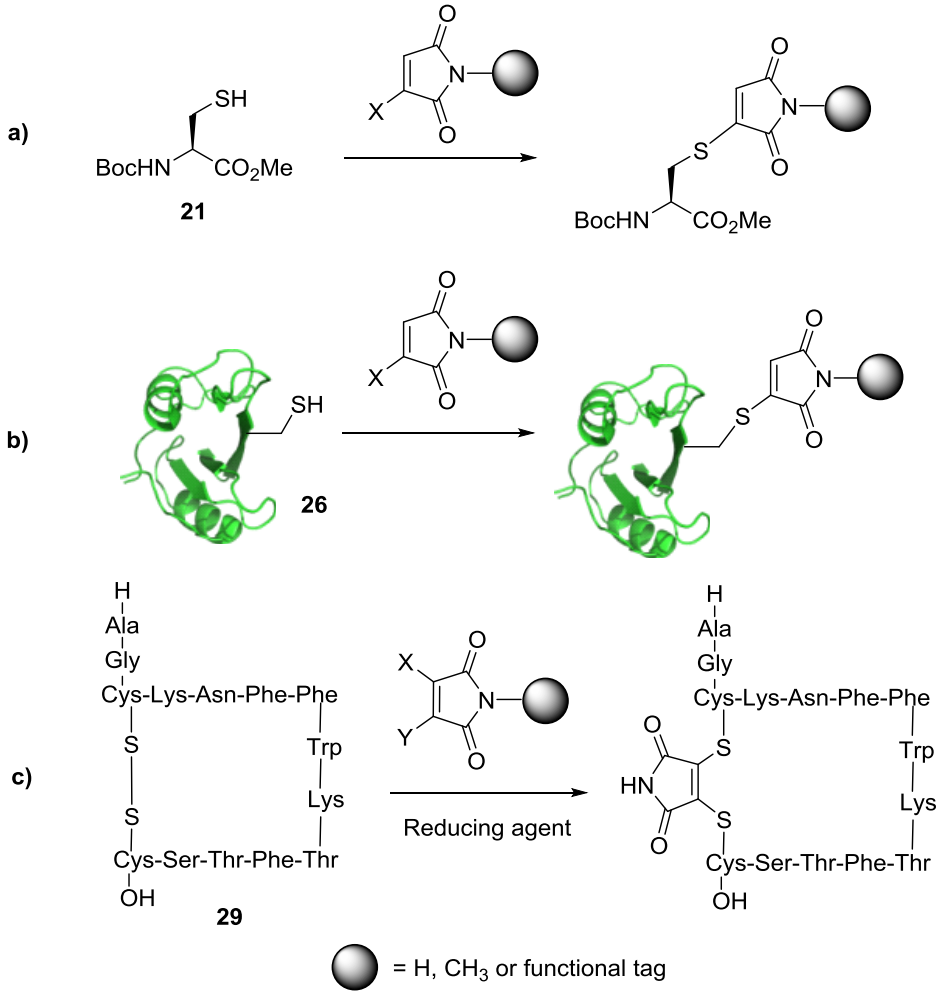
R_1 = H or CH_3
X = halogens, SOR, SOAr, SO_2R,SO_2Ar, OAr, OR, NR, NAr, SR, SAr
Y = same as X or another leaving group

Fig. 7. Selected targeted mono- and disubstituted maleimide analogues

It is expected that the reactivity of these analogues towards thiols and also the regioselectivity of the reaction is dictated by the substituents on the double bond. When predicting the influence of a substituent, several effects need to be considered: sterics, electronic effects (inductive and mesomeric) and leaving group ability. Therefore by carefully choosing the substituents, a range of reagents with tuned reactivity and properties will be accessed.

Once synthesised, the reactivity of monosubstituted maleimide analogues towards cysteine will be tested on the model systems used previously in our group: firstly on the protected cysteine **21** (Scheme 38a) and then on the single cysteine mutant Grb2 SH2 (L111C) **26** (Scheme 38b).

Disubstituted maleimide analogues are potential cystine labelling reagents, therefore the model that will be used is somatostatin **29** (Scheme 38c). The applicability of the reagents for both the stepwise and *in situ* disulphide bridging will be studied.



Scheme 38. Predicted reaction profiles for monosubstituted analogues towards (a) *N*-Boc protected methyl ester cysteine **21**; (b) Grb2 SH2 (L111C) protein and of (c) disubstituted analogues towards somatostatin

The nitrogen atom represents a useful point of attachment for useful functionalities such as fluorophores, PEG chains, drug molecules and affinity tags.

An electron withdrawing group on the double bond is expected to activate the reagent towards thiol attack while an electron donating group is expected to have the opposite effect. This means that a monosubstituted maleimide bearing an electron withdrawing substituent (e.g. sulfoxy, sulfonyl) should react faster with thiols compared to a monosubstituted maleimide bearing an electron donating substituent (e.g. amino, alkoxy). Furthermore, the nature of substituent is expected to have an

effect on the regioselectivity of the reaction with thiols. Fig. 8 shows the effects taking place in the molecule, in the presence of an electron withdrawing group (EWG) and of an electron donating group (EDG) respectively. The former lowers the electron density in the β-position, rendering the β-carbon atom more electrophilic. Consequently, the thiol is expected to add β with respect to the substituent. EDGs are expected to have the opposite effect, deactivating the β-position and thus directing the nucleophile attack α with respect to the substituent.

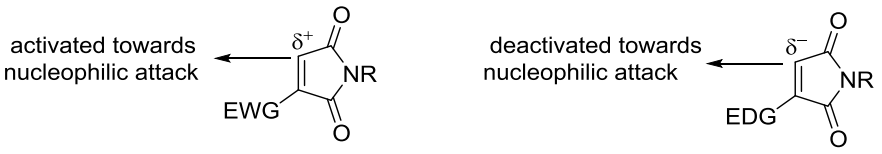


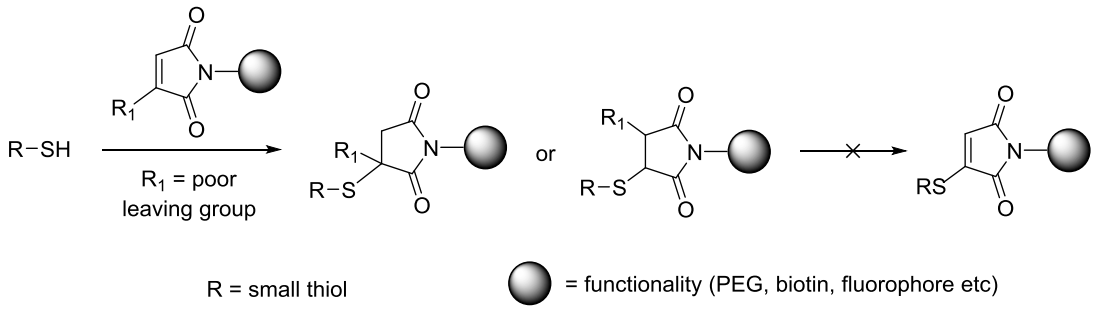
Fig. 8. Electronic effects induced by the substituents on the double bond

Table 1 summarises the electronic effects that the substituents explored in this work are expected to display on the maleimide core.

Substituent	Mesomeric effect	Inductive effect
Halogens	Electron donating	Electron withdrawing
Amino	Electron donating	Electron withdrawing
Alkoxide	Electron donating	Electron withdrawing
Aryloxide	Electron donating	Electron withdrawing
Sulfonyl	Electron withdrawing	Electron withdrawing
Thiol	Electron donating	Electron withdrawing
Sulfoxy	Electron withdrawing	Electron withdrawing

Table 1. Electronic effects of various substituents

Other factors that are expected to influence the rate of thiol addition are size (bulkier groups are expected to hinder the approach of thiols) and leaving group ability (this can be correlated to the value of the pK_a). While sulfoxy, halides are good leaving groups, alkoxy and amino groups are poor leaving groups. Thiol attack onto maleimides bearing poor leaving groups is expected to lead to the addition product only (Scheme 39).



Scheme 39. Expected reaction profile for the thiol addition to monosubstituted maleimides bearing a poor leaving group

By performing reactions between the synthesised analogues and the model systems mentioned above **21**, **26** and **29** we expect to as asses properties that are critical in bioconjugation: stability under physiological conditions, water-solubility and cross-reactivity with other reagents. This will allow the selection of the most suitable reagents depending on the requirements of the envisaged application.

Novel applications of the new analogues in the area of site-selective chemical modification of proteins will also be investigated, with a particular focus on therapeutic proteins.

II Results and Discussion

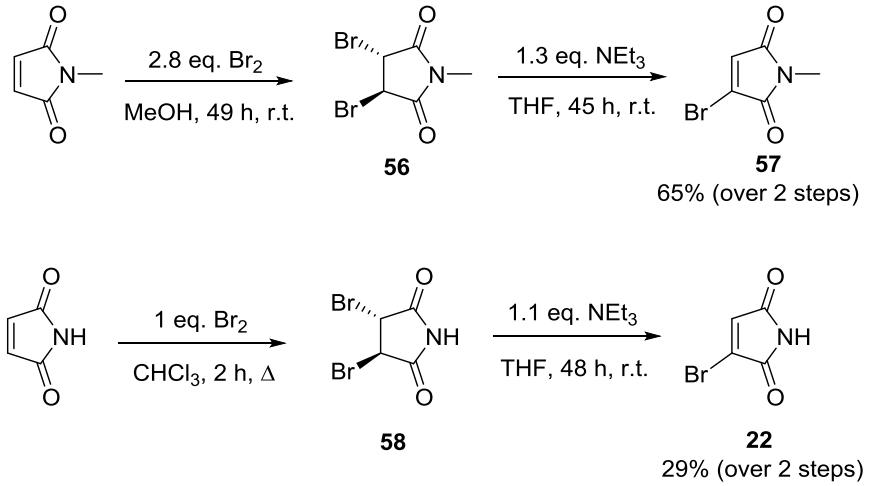
This chapter will be divided in five parts.

The first part, Chapter II.1, concerns the synthesis of the library of compounds. The synthetic section will be followed by Chapter II.2 dealing with the investigation of the utility of the novel reagents for the site-selective cysteine labelling and disulphide bridging. The next section, Chapter II.3, contains the kinetic study which was designed to quantitatively compare the reactivity of the monosubstituted maleimides. Chapter II.4 introduces the use of phenoxymaleimides for achieving an efficient succinimide bridging of disulphide bond containing biomolecules. The scope and optimization of this novel labelling strategy, together with a novel synthetic route for the nitrogen functionalisation of phenoxymaleimides will be discussed. Finally, Chapter II.5 reports on a novel dual labelling of disulphide containing biomolecules. Its scope and optimization will be discussed.

II.1 Synthesis of the library of compounds

II.1.1 Synthesis of bromomaleimides

Whilst dibromomaleimide and *N*-methyl dibromomaleimide are commercially available, the monosubstituted bromomaleimides had to be synthesised. The synthesis was first attempted using the method previously established in our lab.[138] This two-step procedure consists of the addition of bromine to the maleimide, followed by base-induced hydrogen bromide elimination (Scheme 40). As soon as the formation of clean dibromosuccinimides **56** and **58** was confirmed by ^{1}H-NMR, they were immediately subjected to the dehydrobromination conditions (1.1 - 1.3 eq. of triethylamine in THF). The synthesis of *N*-methyl bromomaleimide **57** following this route was straightforward, affording the desired *N*-methyl bromomaleimide **57** in a satisfactory overall yield (65%). However, this was not the case for bromomaleimide **22**. Despite several attempts, the reaction was unsuccessful several times, yielding no more than 29% as an overall yield, which decreased upon scaling-up.

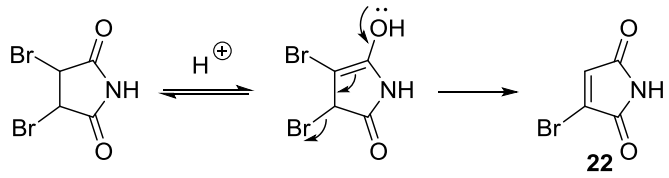


Scheme 40. Synthesis of bromomaleimides **57** and **22** using the classical procedure

It was assumed that the side reaction preventing facile synthesis of **22** is oligomerisation or polymerisation, triggered by the deprotonation imide in the ring (pK_a~10.8),[176] which is plausible considering the basic reaction conditions. Once deprotonated, the negatively charged nitrogen could act as a nucleophile in a conjugate addition to another maleimide molecule and this sequence could continue, leading to oligomers and polymers. Another possibility is that the resulting enolate can add to another maleimide. Triethylamine and DBU were tested as

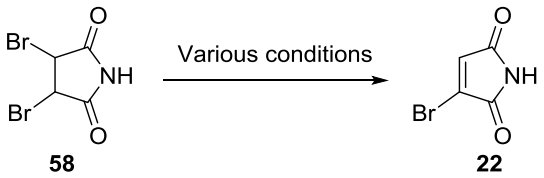
bases, as well as a new strategy consisting of the initial protection of the amino group with a Boc group, chosen based on its stability under basic conditions.

It was then decided to take a closer look at the previously employed procedure and purify the intermediate succinimide **58**, in order to avoid the possibility that any impurities from the first step interfered with the elimination step. To our surprise, the result of flash chromatography on silica gel of the crude dibromosuccinimide was a 1 : 4 (molar ratio, based on ^{1}H-NMR) inseparable mixture of the dibromosuccinimide **58** and the bromomaleimide **22**. It was postulated that silica, due to its acidic character, promotes enolisation of dibromosuccinimide, leading to elimination of hydrogen bromide and subsequent formation of bromomaleimide **22** (Scheme 41).



Scheme 41. Postulated mechanism for the formation of bromomaleimide **22**

It was decided to take advantage of this result and try to establish a new methodology for the synthesis of the bromomaleimides *via* an acid induced elimination of hydrogen bromide. Fig. 9 summarises some of the conditions tested. The transformation did not take place in the presence of a catalytic amount of acid (0.1 eq. *p*-toluenesulfonic acid) or even with a stoichiometric amount of base - silver acetate. The starting material was fully recovered in both cases. Silica, probably due to a combination of the effects of the acidic and basic sites present in its structure, gave satisfactory results even when the reaction was scaled-up. However, the procedure was very long (entry 4, Fig. 9). Gratifyingly, the combination of using 3 eq. of sodium acetate and acetic acid as solvent, gave excellent results (82% isolated yield) in short reaction times (3 h), (entry 5, Fig. 9). The addition of base is needed to trap the bromide as sodium bromide salt; it was shown that otherwise the hydrogen bromide would inhibit reaction.



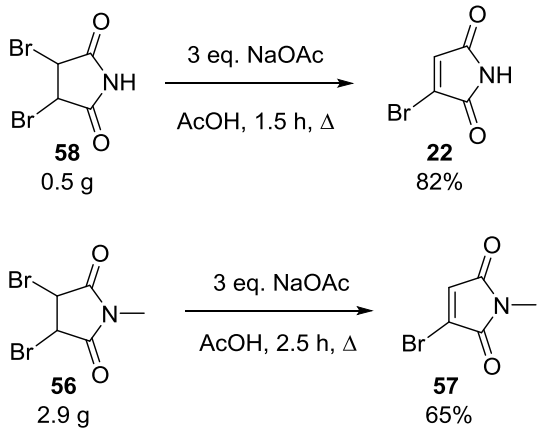
58 **22**

Entry	58 (mg)	Reagent (eq.)	Solvent	Reaction time, temperature	22 (%)
1	100	AgOAc (1.eq.)	EtOAc	15 h @ r.t.	0[a]
2	100	*p*-TsOH (0.1 eq.)	EtOAc/CH_2Cl_2	15 h @ r.t.	0[a]
3	100	*p*-TsOH (0.1 eq.)	Toluene	2 h @ 100 °C, then 13 h @ r.t.	0[a]
4	500	Silica (10g)	CH_2Cl_2	48h @ r.t., then 6 h on the rotaevaporator @ 50 °C	91
5	500	NaOAc (3 eq.)	Acetic acid	1.5 h @ reflux	82

[a] Starting material did not react, based on ^{1}H-NMR of crude mixture at the end of the reaction

Fig. 9. Optimisation of the synthesis of bromomaleimide **22**

This new methodology was applied to both substrates and gave good results on a multi-gram scale (Scheme 42).

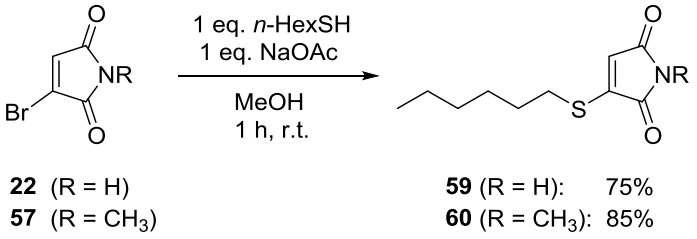


Scheme 42. Novel route for the synthesis of bromomaleimides **22** and **57**

In conclusion, a fast, reliable and scalable methodology was developed for the synthesis of bromomaleimides. Bromomaleimide **22** is of considerable importance due to the possibility of functionalisation on the nitrogen atom, e.g. *via* a Mitsunobu reaction. However, for the purpose of building a library of compounds, the main target will be the *N*-methyl maleimides (both mono- and disubstituted). This avoids complications that may arise from the unprotected secondary imine.

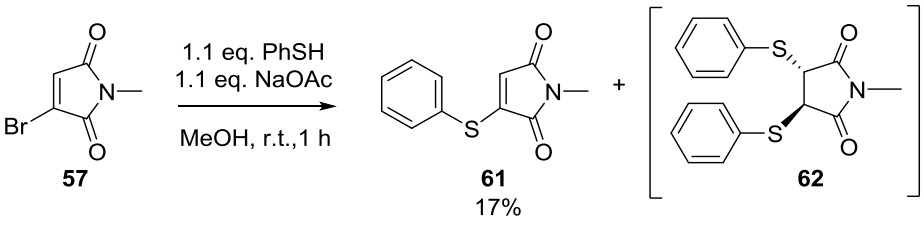
II.1.2 Synthesis of thiomaleimides

The synthesis of thiomaleimides **59** and **60** was achieved according to literature procedures,[177] *via* the conjugate addition of the *n*-hexanethiol to bromomaleimide **22** and *N*-methyl bromomaleimide **57** respectively, in the presence of sodium acetate in methanol (Scheme 43).



Scheme 43. Synthesis of thiomaleimides **59** – **60**

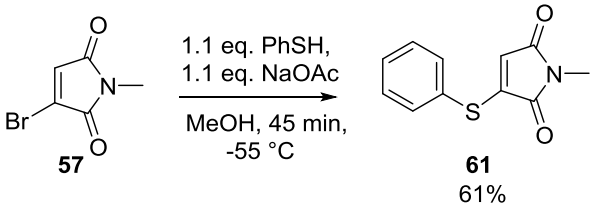
Treatment of *N*-methyl maleimide **57** with thiophenol under the same conditions as above, afforded the desired product **61** in a much lower yield (17%, isolated yield), the major product being the double addition product, dithiosuccinimide **63** (Scheme 44). Compound **62** was not isolated but its identity is confirmed by the ^{1}H-NMR of the fraction containing impure **61**. The formation of the double addition product suggests that the addition of thiophenol to thiomaleimide **61** is as fast if not faster than the addition to the starting material, bromomaleimide **57**. This outcome constrasts with the single thiol addition observed for the formation of thiomaleimide **60** and an explanation can be proposed by the relative contributions of the sulphur atoms to the electronics of the maleimide ring. In the case of thiomaleimide **60** the sulphur lone pair is readily available leading to mesomeric donation into the ring, deactivating it as conjugate acceptor. In the case of thiomaleimide **61** the sulphur lone pair is conjugated with the aromatic ring, reducing its mesomeric effect into the maleimide and thus retaining its electrophilicity.



Scheme 44. Synthesis of thiolmaleimide **61** *via* a conjugate addition reaction

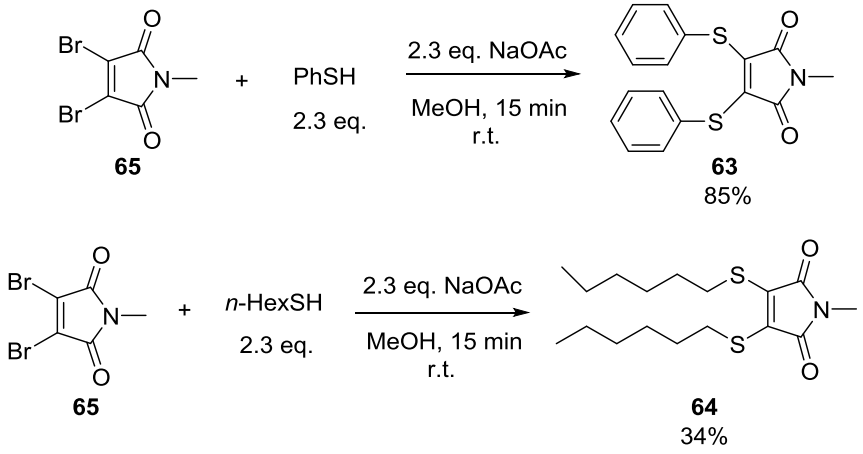
With the aim of reducing the rate of formation of the diaddition product **62**, the addition of thiophenol to *N*-methyl bromomaleimide **57** was performed at various temperatures between -70 °C and 20 °C. The desired product was obtained, as

confirmed by ^{1}H-NMR, without dithiosuccinimide **62**, at a temperature of -55 °C. Following the optimised protocol, thiomaleimide **61** was obtained in an optimised yield of 61%, the rest being unreacted starting material as seen in the ^{1}H-NMR of the crude mixture (Scheme 45).



Scheme 45. Optimised synthesis of the thiomaleimide **61**

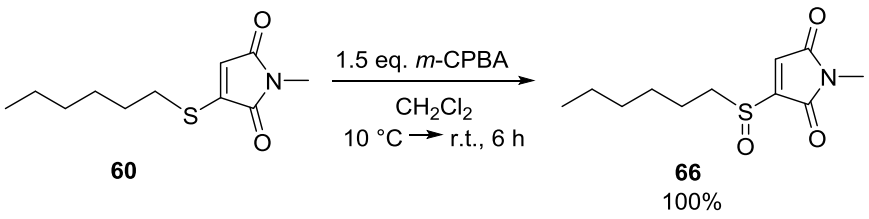
The corresponding dithioether maleimides **63** and **64** were also synthesised in satisfactory yields, *via* the conjugate addition of the corresponding thiol to the commercially available *N*-methyl dibromomaleimide **65** (Scheme 46). The conditions for these reactions have been adapted from a literature procedure.[142]



Scheme 46. Synthesis of dithiomaleimides **63** and **64**

II.1.3 Synthesis of sulfoxymaleimides

With thiomaleimides **60**, **61, 63** and **64** in hand, we set out to synthesise the corresponding sulfoxy analogues. This was attempted using *m*-chloroperbenzoic acid (*m*-CPBA) as the oxidising agent. It was shown that side reactions can be avoided by adding the oxidising agent dropwise and by performing the addition at -10 °C. In this way, sulfoxymaleimide **66** was obtained quantitatively from thiomaleimide **60** (Scheme 47).



Scheme 47. Oxidation of thiomaleimide **60** to sulfoxymaleimide **66**

Despite literature precedent stating a quantitative yield for the synthesis of sulphoxymaleimide **67,**[178] when this protocol was followed, the crude sulphoxymaleimide product **67** contained approximately 15% (molar percent, based on ^{1}H-NMR) of an unidentified product. The formation of the product is confirmed by a peak at 7.18 ppm, corresponding to the hydrogen on the double bond and by the peak at 2.98 ppm, corresponding to the methyl group on the nitrogen (Fig. 10). Attempts to purify it by flash chromatography and by washing it with sodium bicarbonate and sodium sulfite failed, due to the instability of product **67** on the silica gel.

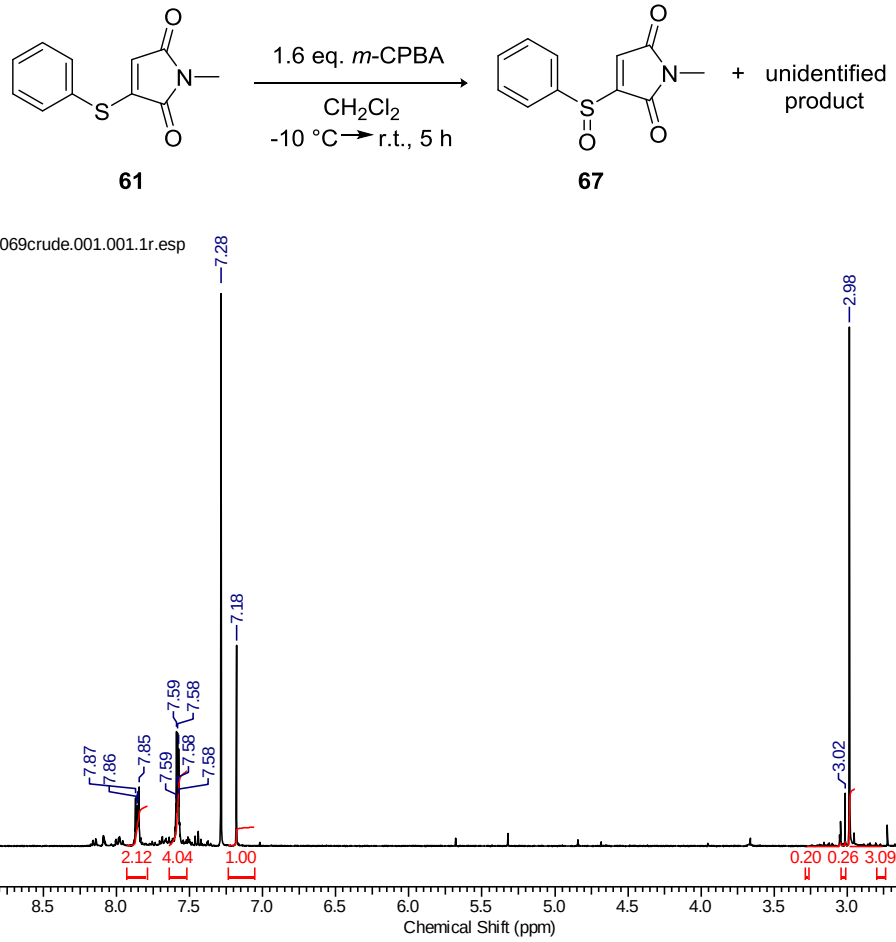
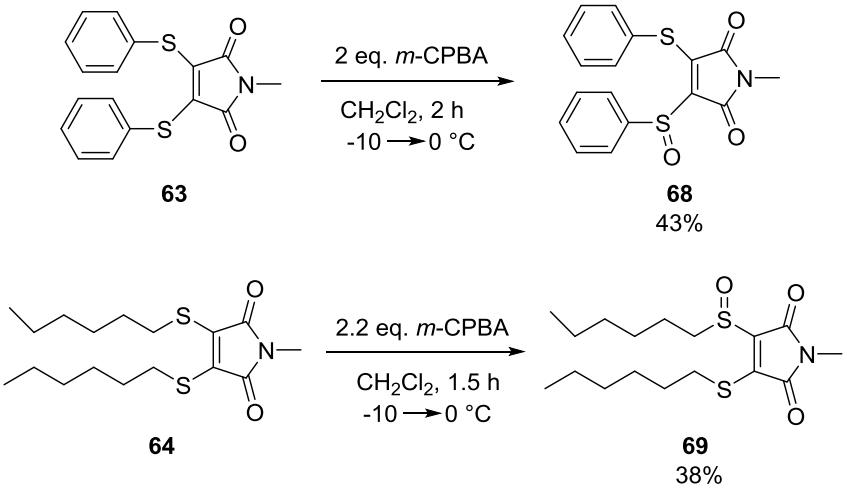


Fig. 10. Oxidation of thiomaleimide **61** and the ^{1}H-NMR spectrum of the resulting crude mixture

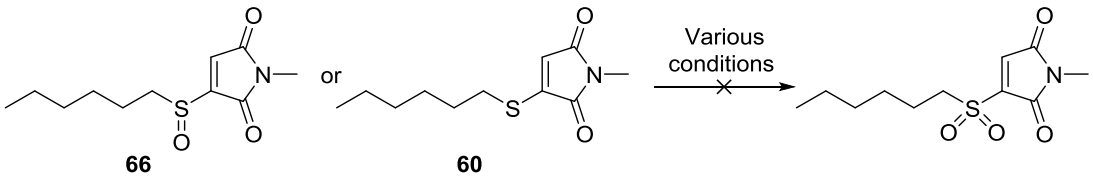
The oxidation of dithiomaleimides **63** and **64** was also performed and led in both cases to the mono oxidised products **68** and **69** (Scheme 48). Our attempts to further oxidise the products by longer reaction times and excess reagent failed as more degradation occurred. Compounds **68** and **69**, however, were stable on silica gel which allowed purification by flash chromatography.



Scheme 48. Oxidation of dithiomaleimides **63** and **64**

II.1.4 Synthesis of sulfonylmaleimides

The first synthetic pathway explored for the synthesis of sulfonylmaleimides was the oxidation of the thioether maleimides. The oxidation of sulphides, using reagents such as H_2O_2, *m*-CPBA, $KMnO_4$, CrO_3/HIO_5 is the most popular method to synthesise sulfones.[179] Firstly, the oxidation of thiomaleimide **60** was attempted using 2 eq. of *m*-CPBA (at 0 °C for the duration of the addition, then at room temperature) but this led to very complex reaction mixture, containing side-products postulated to be derived from attack of the oxidising agent on the double bond (Fig. 11). The reaction was attempted again, starting from sulfoxymaleimide **66** to which 1 eq. *m*-CPBA was added but that too was unsuccessful. A different approach, based on H_2O_2 and ammonium heptamolibdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_20$) as catalyst,[179] also failed to give the desired product. The analysis of the crude mixtures could not be properly quantified due to the instability of some of the products (sulfoxymaleimides, epoxides) on silica gel.

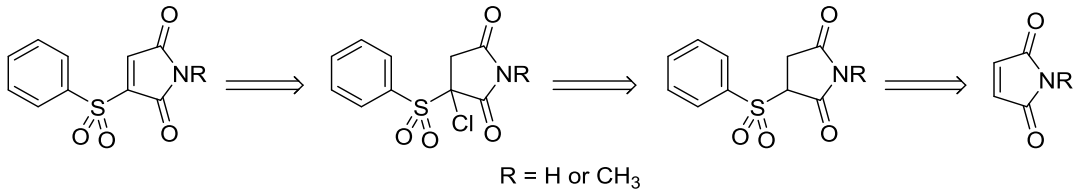


66 60

Substrate	Oxidising agent, (eq)	Eq.	Solvent	Time (h)	Temperature
60	*m*-CPBA	2	CH_2Cl_2	21	0 °C → r.t.
66	*m*-CPBA	1	CH_2Cl_2	10	0 °C → r.t.
60	H_2O_2, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	4	MeOH	0.5	r.t.

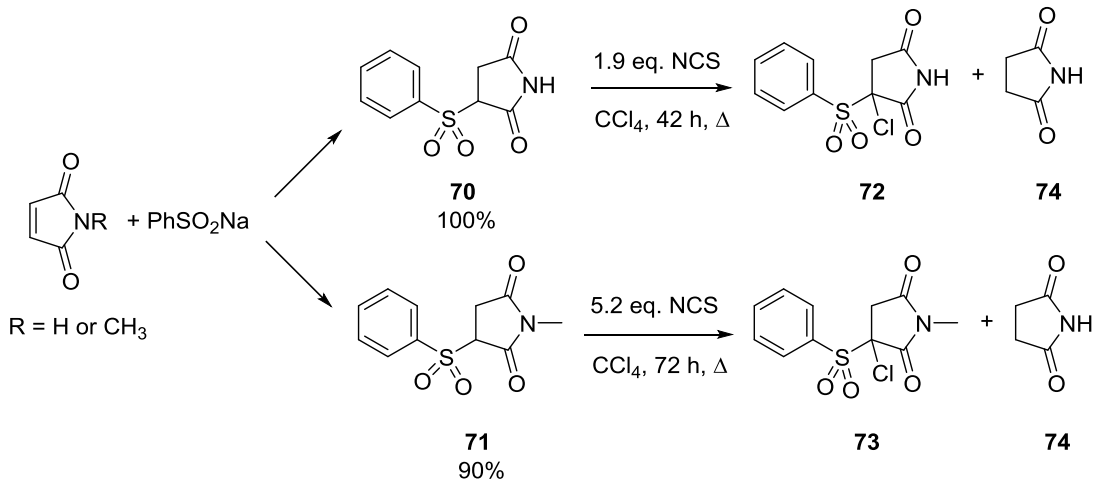
Fig. 11. Unsuccessful oxidation conditions

Scheme 49 outlines the retrosynthetic analysis for the synthesis of sulfonemaleimides wherein the key step is the base-induced dehalogenation of chlorinated sulfonylsuccinimide. These can be obtained by chlorination of the commercially available maleimide or *N*-methyl maleimide respectively.



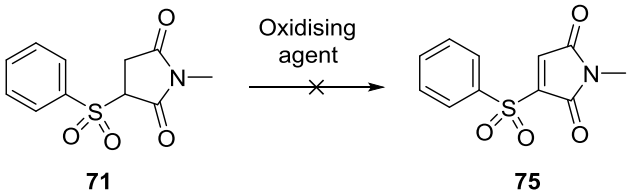
Scheme 49. Retrosynthetic route to sulfonylmaleimides

First the Michael addition of sodium benzenesulfinate to maleimide and *N*-methyl maleimide was explored (Scheme 50).[180] The reaction is reversible but in a buffered aqueous medium, the formation of the product is favoured by its precipitation. Once the sulfonylsuccinimides **70** and **71** were obtained, they were chlorinated with *N*-chlorosuccinimide in carbon tetrachloride. Chlorination took place only in the α-position with respect to the sulfonyl. However, solids **72** and **73** were obtained as an inseparable mixture with succinimide **74** - the by-products of this reaction - and the unreacted chlorinating agent. Unfortunately, purification by flash chromatography or by recrystallization did not allow isolation of the desired products.



Scheme 50. Synthesis of the chlorinated sulfonylsuccinimides **72** and **73**

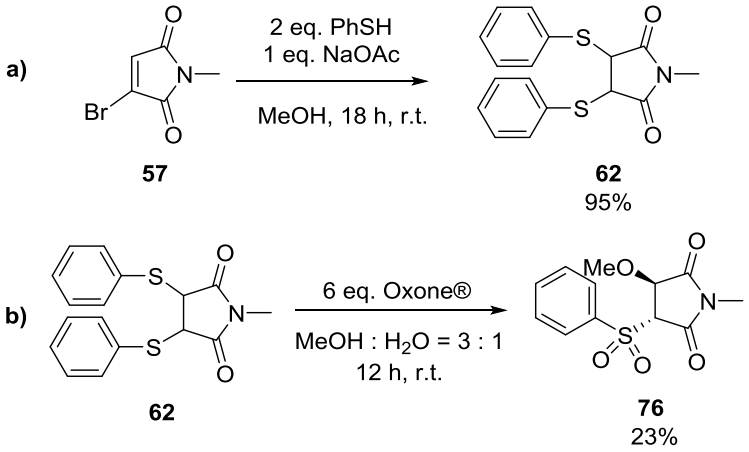
Another alternative to get sulfonemaleimides is to oxidise the sulfonylsuccinimides **70** and **71** to the corresponding maleimides by using an oxidising agent such as 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), MnO_2 or SeO_2. A literature search on this topic yielded several papers describing DDQ oxidations of indolesuccinimides to the corresponding maleimides.[181,182] Also, Duan *et al.* reported on the oxidation of a phenyl substituted succinimide using a mixture of DEAD and K_2CO_3.[183] MnO_2 is a mild oxidising agent,[184] used for oxidations of activated bonds such as benzylic or allylic, while SeO_2 is a stronger oxidising agent used successfully in the synthesis of various natural products.[185,186] All of these protocols were tested and were found to be unsuccessful, the succinimide remaining inert under the reaction conditions (Scheme 51).



Scheme 51. Attempts to oxidise succinimide **71**

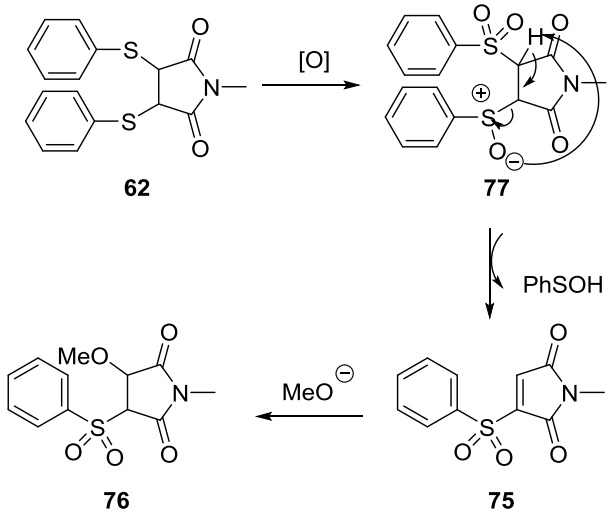
A final oxidation reagent that was investigated was investigated was Oxone®. It was decided to first test the oxidising ability of this reagent on dithiosuccinimide **62**, as this avoids possible side reactions involving the double bond. Dithiosuccinimide **62** was obtained *via* conjugate addition of 2.2 eq. thiophenol to bromomaleimide **57** in excellent yield (95%), (Scheme 52a). The reaction between dithiosuccinimide **62** and Oxone® was performed in a mixture of methanol and

water, to ensure the solubility of all reactants. Surprisingly, instead of the expected di-oxidised product, product **76** was isolated in 23% yield (Scheme 52b).



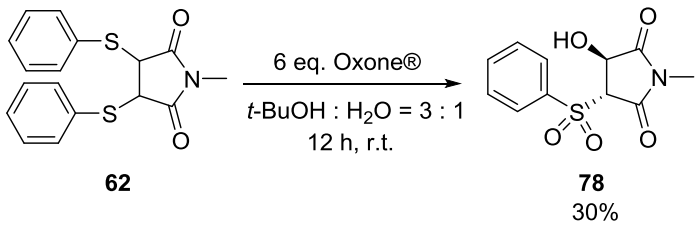
Scheme 52. a) Synthesis of dithiosuccinimide **62** and b) its oxidation with Oxone®

One of the postulated mechanisms for the formation of **76** is depicted in Scheme 53. Once intermediate **77** is formed, the sulfoxy group abstracts the acidic proton α to the carbonyl, leading to sulfonylmaleimide **75**. This intermediate is too reactive to be isolated and in the presence of excess methanol, undergoes attack on the double bond to afford the isolated product **76**.



Scheme 53. Postulated mechanism for the formation of **76**

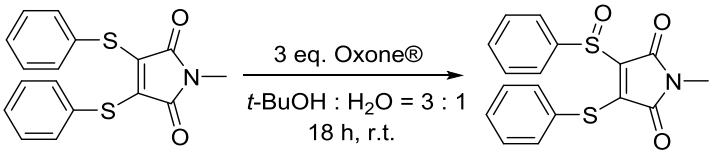
In order to avoid the attack of the methoxy group, the reaction was performed in a mixture of water and *tert*-BuOH (Scheme 54). It was expected that the more bulky *tert*-butoxide group would not react with sulfonylmaleimide **75**. This assumption proved correct however, in this case intermediate **75** reacted with the other solvent - water - as certified by the isolated product, **78**.



62 **78** 30%

Scheme 54. Oxidation of dithiosuccinimide **62** with Oxone®

As the α-proton to the carbonyl in compound **62** was responsible for the formation of side products **76** and 7**8**, it was decided to attempt this reaction on dithiomaleimide **63** (Scheme 55). This time the number of equivalents of oxidizing agent was reduced to 3 due to the risk of the Oxone® attack on the double bond. Oxidation took place only on one of the sulphur atoms, giving compound **68** in 49% yield after 18 h at room temperature. This same product was obtained when the oxidizing agent used was *m*-CPBA, suggesting that oxidation of both sulphur atoms is disfavored.



63 **68** 49%

Scheme 55. Oxidation of dithiomaleimide **63** with Oxone®

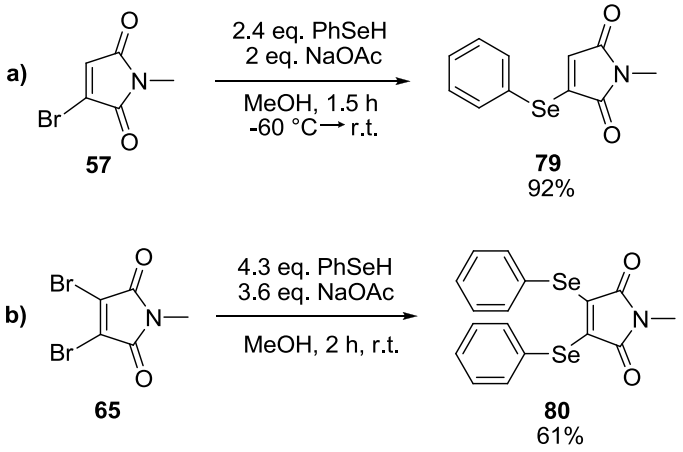
In conclusion, sulfonylmaleimides proved very difficult to synthetise. The reaction with Oxone® suggests that these reagents are extremely reactive, due to the sulfonyl group which strongly activates the double bond towards nucleophilic attack. Even more, this provides the proof that they are unstable in water therefore they would be unsuitable as protein modification reagents.

II.1.5 Synthesis of selenomaleimides

While the diselenomaleimides had been previously synthesised in our group, [187] a method was required for the synthesis of the monosubstituted analogue **79**. In analogy with thiomaleimide **61**, we envisaged the risk of double addition; consequently, the optimised conditions for the synthesis of **61** were employed. After completion of the addition of a stoichiometric amount of *N*-methyl bromomaleimide **57** to a solution of benzeneselenol in methanol (45 min) at -55 °C and another 15

min of stirring at room temperature, the desired product **79** was obtained in an excellent yield (92%), (Scheme 56a).

The synthesis of the diselenomaleimide **80** from *N*-methyl dibromomaleimide **65** was less efficient (Scheme 56b). The large excess of benzeneselenol used is due to the poor quality of the reagent (which during storage oxidises to give the diselenide) and should not be associated with a decrease reactivity of the selenate ($PhSe^-$) towards the double bond in the maleimide ring.

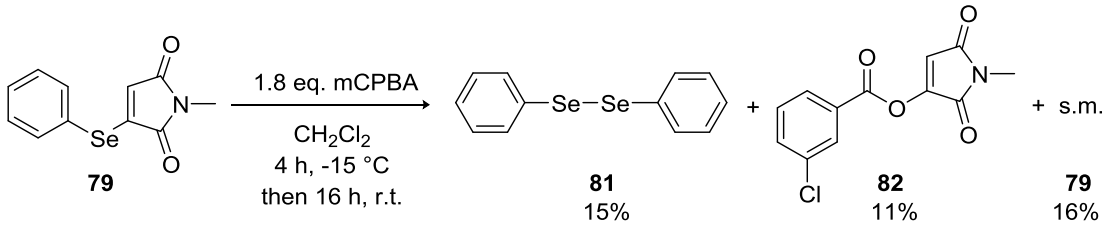


Scheme 56. Synthesis of selenomaleimides **79** and **80**

II.1.6 Synthesis of selenoxymaleimides

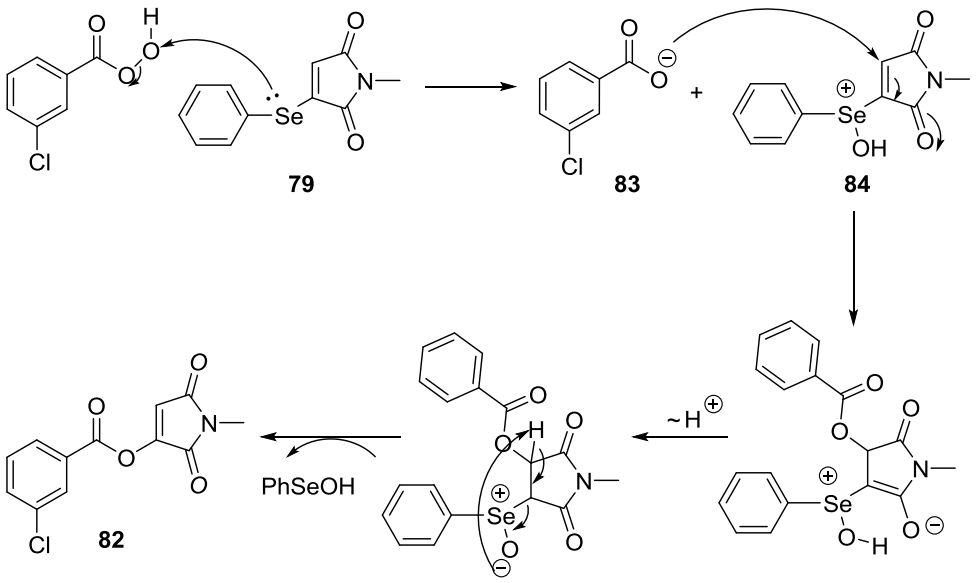
With the aim of further activating the maleimide reagents towards thiol attack, the oxidation of selenomaleimides **79** and **80** was attempted. The oxidising reagent of choice was *m*-CPBA and the reactions were performed at -15 °C for 4 h and then room temperature, overnight.

Unfortunataly, the desired oxidation products could not be obtained. When *m*-CPBA was added to the selenomaleimide **79**, a mixture of diselenide **81**, unreacted starting material **79** and the unexpected benzoatemaleimide **82** was formed (Scheme 57).



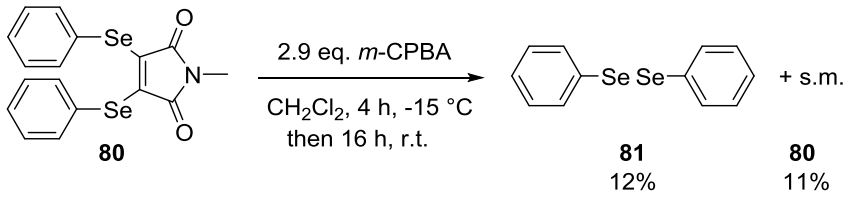
Scheme 57. Oxidation of selenomaleimide **79**

The postulated mechanism leading to the formation of compound **82** is shown in Scheme 58. First, the *m*-CPBA oxidises the selenomaleimide **79** *via* the classical mechanism. The resulting benzoate ion **83** attacks the double bond in **84** and displaces the highly reactive selenoxide leaving group to give benzoatemaleimide **82** and PhSeOH. To our knowledge, this is the first example of a benzoatemaleimide.



Scheme 58. Postulated mechanism for the formation of benzoatemaleimide **82**

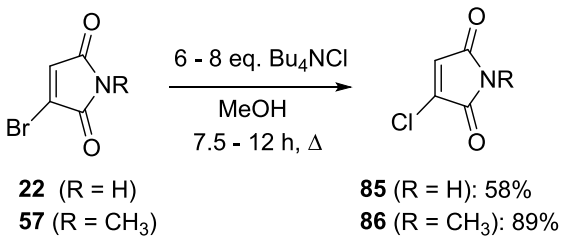
In the case of diselenomaleimide **80**, no trace of the oxidation product could be isolated (Scheme 59). Flash chromatography afforded unreacted starting material **80** (11%) and diselenide **81** (12%), indicating extensive degradation.



Scheme 59. Oxidation of diselenomaleimide **80**

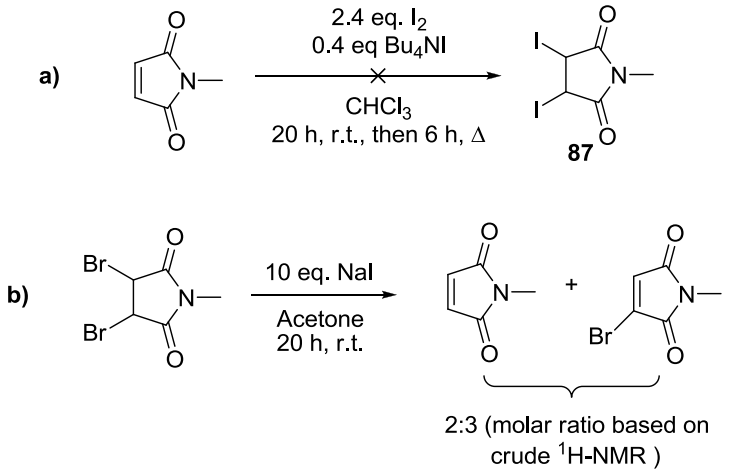
II.1.7 Synthesis of halomaleimides

Chloromaleimides **85** and **86** were obtained by conjugate addition of tetrabutylammonium chloride to the corresponding bromomaleimides, **22** and **57**. The transformations required excess chlorinating agent (6 eq.) and refluxing the reaction mixture for 7 - 12 h, though the products were obtained in good yields (Scheme 60).



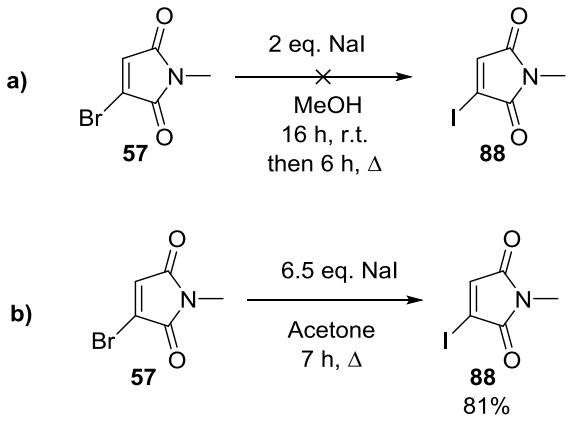
Scheme 60. Synthesis of chloromaleimides **85** and **86**

For the synthesis of the iodomaleimides, two procedures were first employed: addition of iodine to maleimide (Scheme 61a) and addition of sodium iodide to dibromosuccinimide using the Finkelstein reaction conditions (Scheme 61b). Both reactions failed to yield the desired diiodosuccinimide **87**: in the case of iodine addition, the maleimide was fully recovered at the end of the reaction, proving to be completely unreactive towards iodine, while for the Finkelstein reaction, the desired bromo-iodo exchange did not take place.



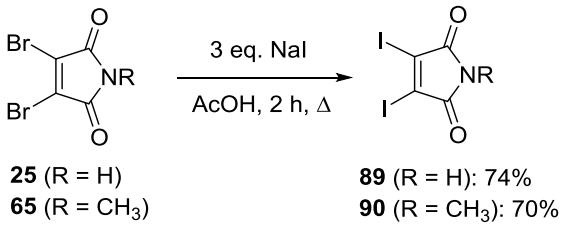
Scheme 61. Attempts to synthesise diiodosuccinimide **87**

Next, the conjugate addition of an iodide source to bromomaleimide **57** was considered. The reaction was initially performed using 2 eq. of sodium iodide in MeOH, but under these conditions bromomaleimide **57** proved to be completely stable (Scheme 62a). A larger excess of NaI (6.5 eq.) and acetone instead as solvent finally lead to the desired product, iodomaleimide **88,** in very high yield (81%), (Scheme 62b).[188] Acetone enables the precipitation of the sodium bromide formed, favouring the formation of **88** by pushing the equilibrium towards the product side.



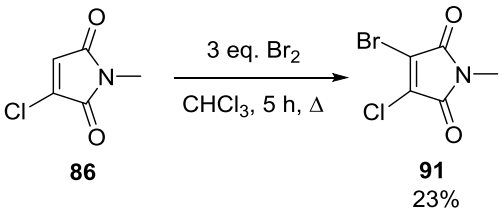
Scheme 62. Conjugate addition of sodium iodide to bromomaleimide **57**

Diiodomaleimides **89** and **90** were synthesised according to a similar literature procedure in 70% and 74% yield (Scheme 63).[189] The products were isolated by filtration after addition of water to the reaction mixture.



Scheme 63. Synthesis of diiodomaleimides **89** and **90**

A mixed halomaleimide was considered to be of interest as well, most importantly because it would give more insight into the regioselectivity of the thiol attack. Bromochloromaleimide **91** was synthesised in one step from chloromaleimide **86** by bromination with 3 eq. of bromine, at reflux (Scheme 64).

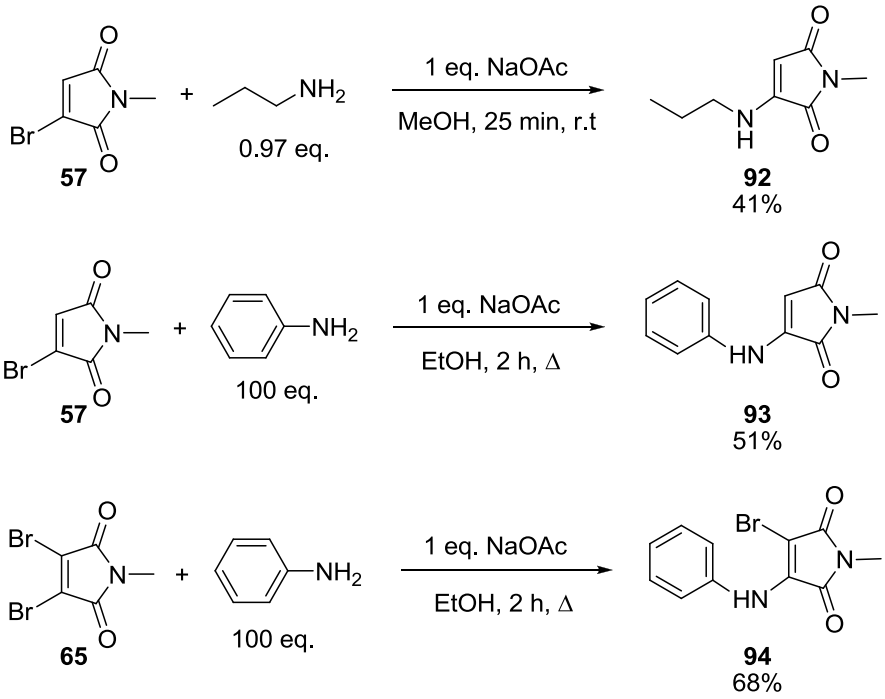


Scheme 64. Synthesis of the mixed halomaleimide **91**

II.1.8 Synthesis of aminomaleimides

Some examples of aminomaleimides were required to complete the proposed maleimide library. *n*-Propylamine and aniline were chosen as representatives for alkyl and aromatic primary amines and reacted with

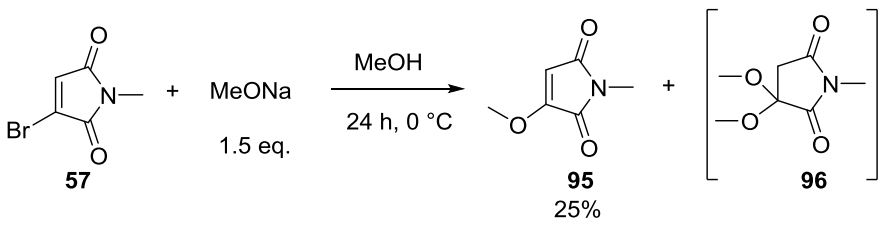
bromomaleimide **57**, in the presence of sodium acetate, according to similar literature procedures (Scheme 65).[177,190] All attempts to displace the bromine with 4-nitroaniline failed, probably due to the electron withdrawing effect of the nitro group. This reduces the nucleophilicity of the amine by decreasing the density of electrons on the amino group.



Scheme 65. Synthesis of aminomaleimides **92**, **93** and **94**

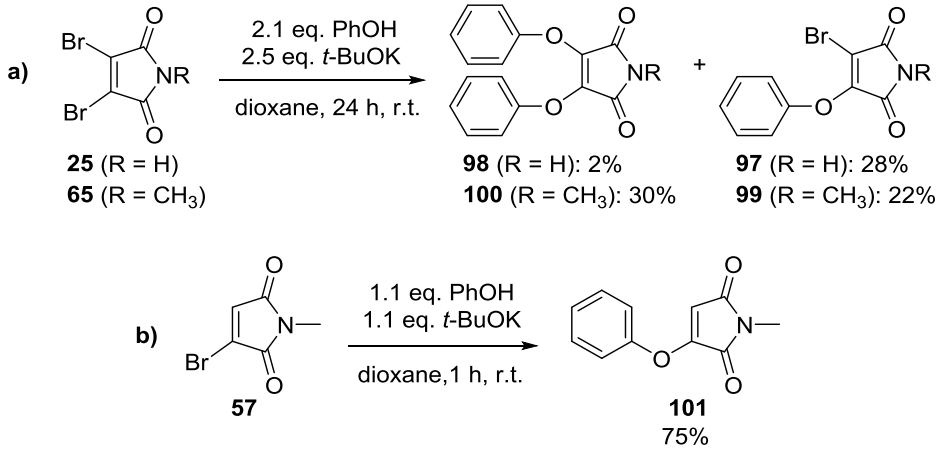
II.1.9 Synthesis of alkoxy- and phenoxymaleimides

Methoxymaleimide **95** was synthesised according to a literature procedure in 25% yield.[191] The low yield is likely due to the formation of the diaddition product, **96**, as indicated by the ^{1}H-NMR of the crude (Scheme 66).The second attack of the methoxy group is expected to take place α to the oxygen in the methoxy group due to the mesomeric electron donating effect of the substituent.



Scheme 66. Synthesis of methoxymaleimide **95**

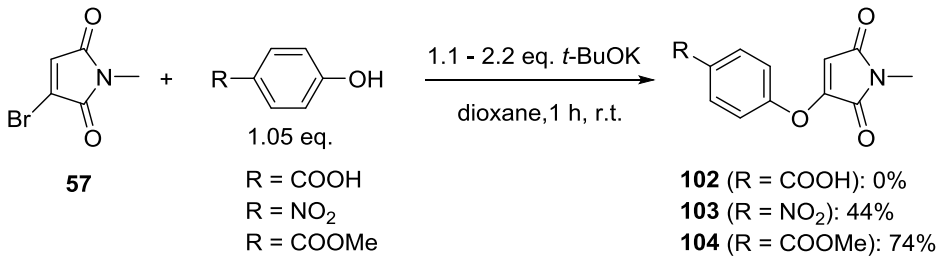
The conjugate addition of phenol to dibromomaleimide has been previously reported in the literature (Scheme 67a).[191] The reaction uses a strong base such as potassium *tert*-butoxide and needs to be performed under dry conditions in order to prevent hydrolysis of the maleimide. The second addition is known[192] to be slowed down by the addition of the first phenoxy group, as this group will deactivate the β-position by increasing its electron density making the attack of a nucleophile more difficult. This may in part account for the isolation of the bromophenoxymaleimide **99** in 22% yield and the low yield of diphenoxymaleimide **100** (30%). In the case of dibromomaleimide **25**, the yields obtained from products **97** and **98** are even smaller, probably due to side-reactions involving the imine. Next, the reactivity of bromomaleimide **57** towards phenol was investigated. Gratifyingly, the phenoxymaleimide product **101** was obtained in a satisfactory yield (75%) after just 1 h at room temperature (Scheme 67b).



Scheme 67. Conjugate addition of phenol to a) dibromomaleimide **25** and **65** and b) bromomaleimide **57**

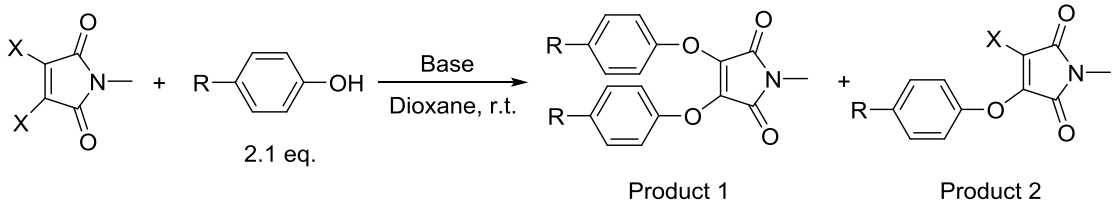
Furthermore, the scope of the reaction of mono- and dibromomaleimides with phenols was explored. Bromomaleimide **57** proved to be reactive towards phenols bearing electron withdrawing groups such as esters and nitro groups, however reaction with 4-carboxyphenol failed to afford the desired product **102** (Scheme 68). The difficulty encountered with the latter reaction arises from the fact that 4-carboxyphenol has two acidic sites, with relative acidities of the carboxylic acid and phenol functional groups of pK_a ~4.48 and ~9.32 respectively.[193] This means the carboxylic acid group will be deprotonated first, followed by the phenol. An excess of *tert*-BuOK (pKa of conjugated acid = 17) was used to ensure double deprotonation. However, the failure of the reaction indicates that the dianion derived

from 4-carboxyphenol is potentially not nucleophilic enough to undergo the addition on the double bond.



Scheme 68. Conjugate addition of phenols to bromomaleimide **57**

In contrast, dibromomaleimide **65** reacted with activated phenols such as 4-methoxyphenol to give diphenoxymaleimide **105** and failed to react with deactivated phenols bearing electron withdrawing groups such as ester and nitro. Even when performed on the more reactive diiodomaleimide **90**, the reaction with methyl 4-hydroxybenzoate yielded the expected diphenoxymaleimide **107** in a very modest yield (13%), accompanied by the monosubstituted product **108** (10%), (Fig. 12). When diiodomaleimide was reacted with 4-nitrophenol, only the monosubstituted maleimide **106** (47%) was formed. These results highlight the decrease in nucleophilicity of the phenol with the installation of more electron withdrawing groups on the phenyl ring.



X	R	Phenol (eq.)	*t*-BuOK (eq.)	Time (h)	Product 1 code, yield (%)	Product 2 code, yield (%)
Br	H	2.1	2.5	24	**98**, 30	**97**, 22
Br	OMe	2.1	2.5	20	**105**, 13	-
I	NO_2	2.2	2.5	64	-	**106**, 47
I	COOMe	2.2	2.5	24	**107**, 13	**108**, 10
Br	COOMe	1.1	1.1	10	-	**109**, 63

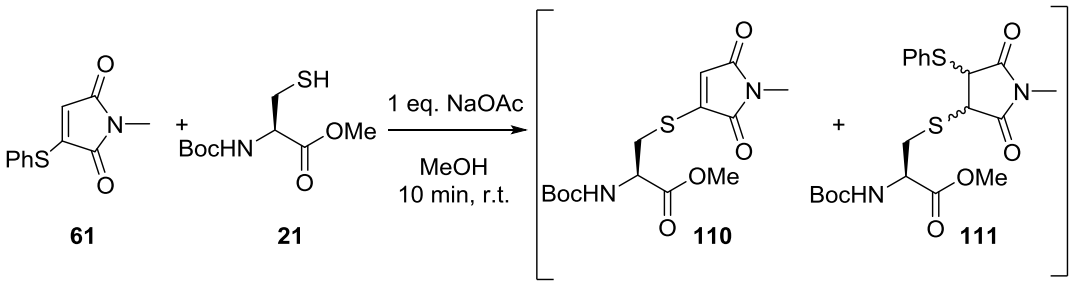
Fig. 12. Addition of phenol to dibromomaleimide **65** and diiodomaleimide **90**

II.2 Investigation of maleimide analogues as reagents for cysteine and disulphide bridging

Having synthesised the maleimide analogues described above, we proceeded to the next stage of the study: the testing of their reactivity towards cysteine. A selection of compounds was tested on our model systems: the protected cysteine **21**, Grb2 SH2 (L111C) **26** and somatostatin **29**. However, given our interest in protein labelling reagents, Grb2 SH2 (L111C) **26** was preferred to *N*-Boc-Cys-OMe **21**. The next section of this report contains the most significant results obtained.

II.2.1 Reactivity of thiomaleimide 61 towards *N*-Boc-Cys-OMe 21

Treatment of the *N*-Boc-Cys-OMe **21** with a small excess (1.1 eq.) of thiomaleimide **61** in the presence of sodium acetate (1 eq.) resulted in a complex mixture of products, which could not be separated by flash chromatography. Scheme 69 shows tentatively proposed products observed in the ^{1}H-NMR of the mixture. Dithiosuccinimide **111** can be present as four diastereoisomers which may serve to complicate their isolation. Given the complexity of the product mixture, it was concluded that this reagent is not useful for cysteine labelling.



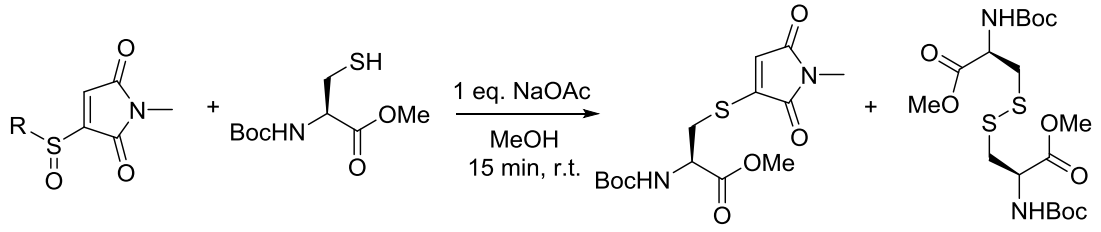
Scheme 69. Reaction of thiomaleimide **61** with the protected cysteine **21**

II.2.2 Reactivity of sulfoxymaleimides

Sulfoxymaleimides bear a strongly electron withdrawing group on the double bond and are therefore expected to be highly reactive towards nucleophiles such as the thiol groups of cysteine.

As for thiophenolmaleimide **61**, the reactivity of sulfoxymaleimide analogues **66** and **67** towards cysteine was first explored on the protected cysteine **21**. The addition of stoichiometric amounts of the two sulfoxymaleimides **66** and **67** resulted in the expected thiomaleimide **110** in 74% and 59% yield respectively. The isolated

products were contaminated with <5% molar percent of the oxidised cysteine **112** which could not be separated from the products by standard chromatographic means. The reactions were very fast, reaching completion within 5 min (Scheme 70).



66 (R = C_6H_{13})
67 (R = C_6H_5)

110 (R = C_6H_{13}): 74%
110 (R = C_6H_5) : 59%

112

Scheme 70. Reaction of sulfoxymaleimides **66** and **67** with the protected cysteine **21**

Having shown that sulfoxymaleimides were successful in the labelling the protected cysteine **21**, the next step was to explore their reactivity in a more relevant biological system. The study of the reactivity of *N*-methyl sulphoxymaleimide **66** towards a cysteine in a protein was performed using Grb2 SH2 (L111C) **26** as a model system. Standard conditions for the Grb2 experiments, which were used throughout the entire study, were a protein concentration of 1 mg/mL in phosphate buffer pH 8 (50 mM) and 1.14 mM reagent stock solutions in DMF. When 100 eq. of the labelling reagent were used, the stock solution was 11.4 mM, to minimise the amount of organic solvent added to the reaction mixture. All reactions were carried out at 0 °C, using an ice bath. The progress of the reactions was monitored by LC-MS, by calculating the ratios between the peak heights of the native and the modified protein in the mass spectrum.

As shown in Fig. 13, rapid (< 5 min) and complete labelling of protein **26** (M = 14170) to give the Grb2-*N*-methyl-thiomaleimide conjugate **113** (M = 14280) was achieved using 4 eq. of sulphoxymaleimide **66**. When 1 and 2 eq. of reagent **66** were used, the conversion after 5 min was only 40% and 64% respectively. Longer reaction times did not improve the conversion, indicating that the starting material had degraded. This could be related to the strong electron withdrawing effect of the sulfoxy group, which renders the maleimide double bond more electrophilic therefore favouring hydrolysis. For the sake of comparison, the LC-MS data for the reaction between the Grb2 SH2 (L111C) **26** and 1 eq. of *N*-methyl bromomaleimide **57** was also plotted.

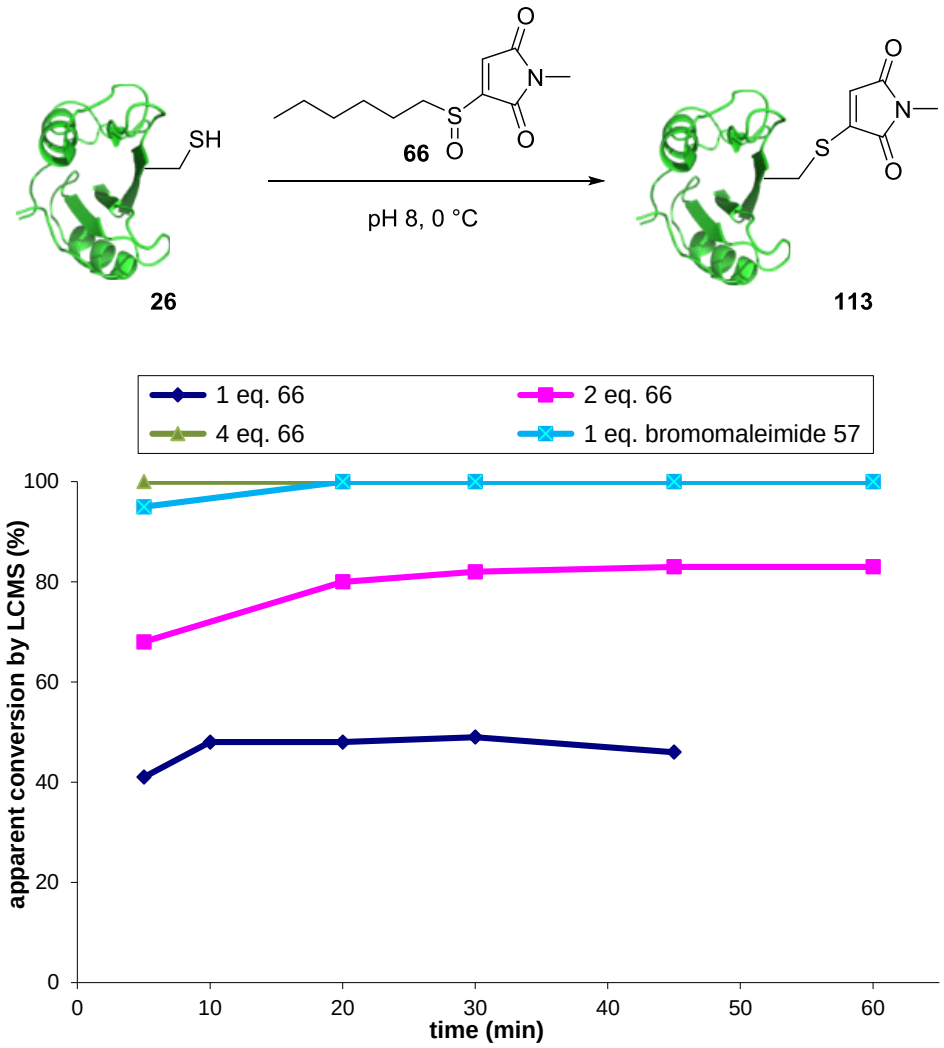


Fig. 13. Labelling of Grb2 SH2 (L111C) with sulfoxymaleimide **66**

To attempt to identify if sulfoxymaleimides were more reactive than bromomaleimides, a competition experiment between sulfoxymaleimide **66** and bromomaleimide **22** was designed. For this Grb2 SH2 (L111C) **26** was added to an equimolar mixture of the two reagents under investigation, using the same concentration of the protein, temperature and buffer as described above. LC-MS of the reaction mixture after 1 min shows that when 2 eq. of each of the reagents were used, the major product is the Grb2-thiomaleimide conjugate **114** (M = 14266), the one derived from bromomaleimide **22** (Fig. 14). The same experiment was repeated with 4 eq. of each of the two reagents led to the reversal of the product distribution, with the Grb2-*N*-methyl thiomaleimide conjugate **113** (M = 14280), the one derived from sulfoxymaleimide **66** as the major product (Fig. 14). This shows that **66** is indeed more reactive than **22** but due to the instability of sulfoxymaleimides in

aqueous environment, it must be used in excess, to allow the reagent to be present in stoichiometric quantity with respect to the protein to be labelled.

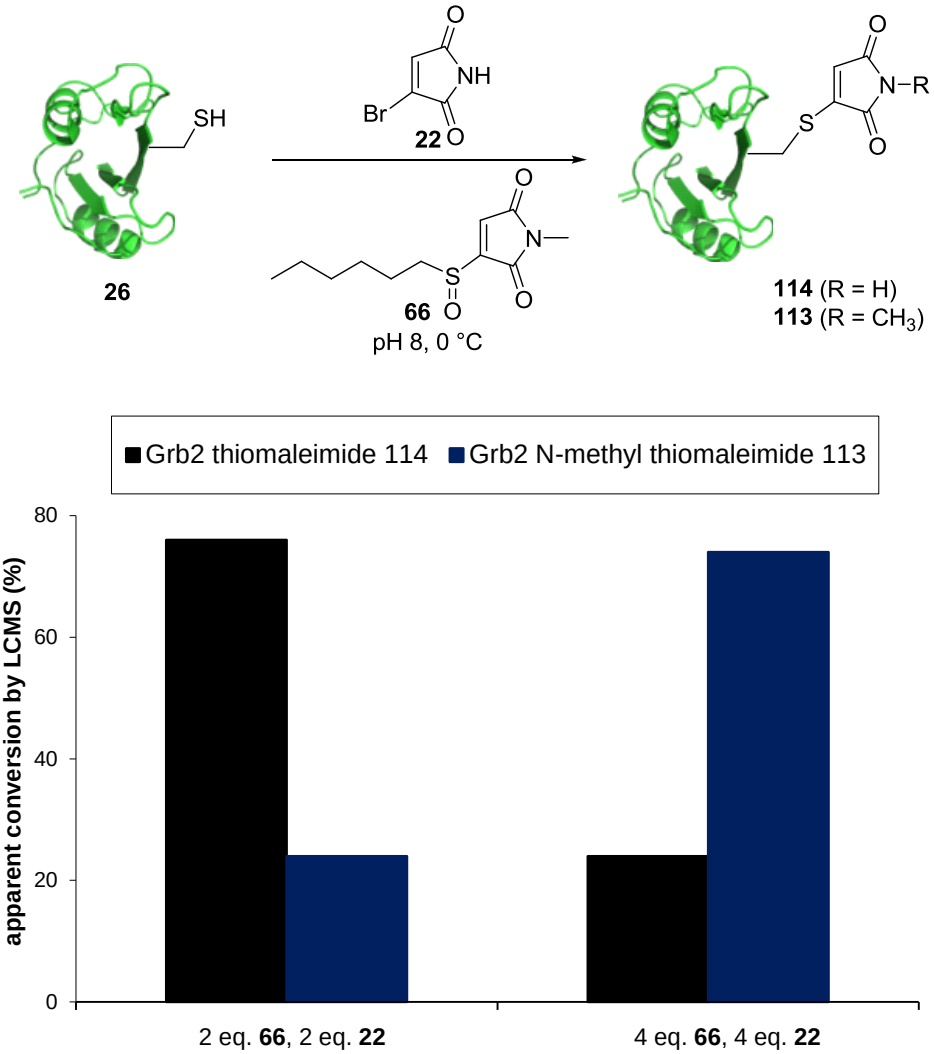


Fig. 14. Competition experiment between bromomaleimide **22** and sulfoxymaleimide **66**

The same comments made for compound **66** apply to compound **67**; with 1 eq. of sulfoxymaleimide **67**, the conversion after 5 min was only 21% and after 1 h, 39% (Fig. 15). From the reaction profiles we can observe that the reaction does not progress any further after 5 - 10 min. This is assumed to be due to the instability of this class of reagents in water. When 4 eq. of maleimide **67** were used, the conversion was 78% after 5 min and 95% after 1 h.

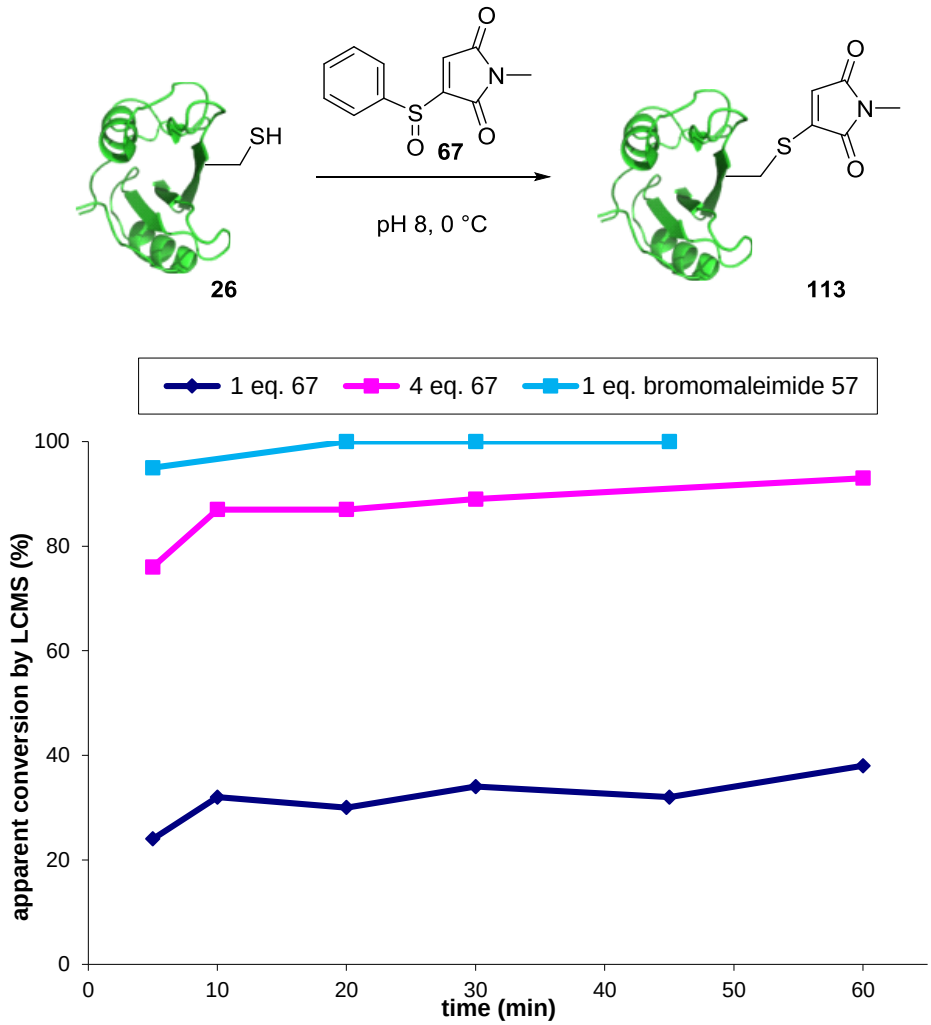


Fig. 15. Labelling of Grb2 SH2 (L111C) with sulfoxymaleimide **67**

In conclusion, the labelling of Grb2 SH2 (L111C) **26** can be achieved with sulfoxymaleimide **66** and **67**. Sulfoxymaleimides appear to display an enhanced reactivity compared to bromomaleimdes. However this is achieved at the expense of their stability in aqueous environment which makes the addition of excess reagent mandatory for pushing the reaction to completion. In terms of practical use, there is no advantage in using sulfoxymaleimides over bromomaleimides. This result might also indicate that reagents faster than bromomaleimides might be too unstable to be used efficiently as protein labelling reagents.

II.2.3 Reactivity of halomaleimides towards Grb2 SH2 (L111C)

Having synthesised three different halomaleimides, the next step was to compare their reactivity towards cysteine and understand the correlation between the halogen substituent and the reactivity of the labelling reagent. The reactions of

the previously described halomaleimides **86**, **57** and **88** with Grb2 SH2 (L111C) **26** were performed under standard conditions (pH 8, 0 °C) and monitored by LC-MS. As shown in Fig. 16, the three halomaleimides display very similar reactivity. All reactions reached 100% conversion within 1 h leading to the Grb2-*N*-methyl thiomaleimide conjugate **113** (M = 14280) as the sole product. The only time point where a significant difference can be seen is the 5 min time point. Based on this, the order of reactivity is: **86** < **57** < **88**. This is in accordance with the leaving group ability of the halogens (Cl < Br< I). A kinetic study was designed in order to compare their reactivity more rigorously (see Chapter II.3).

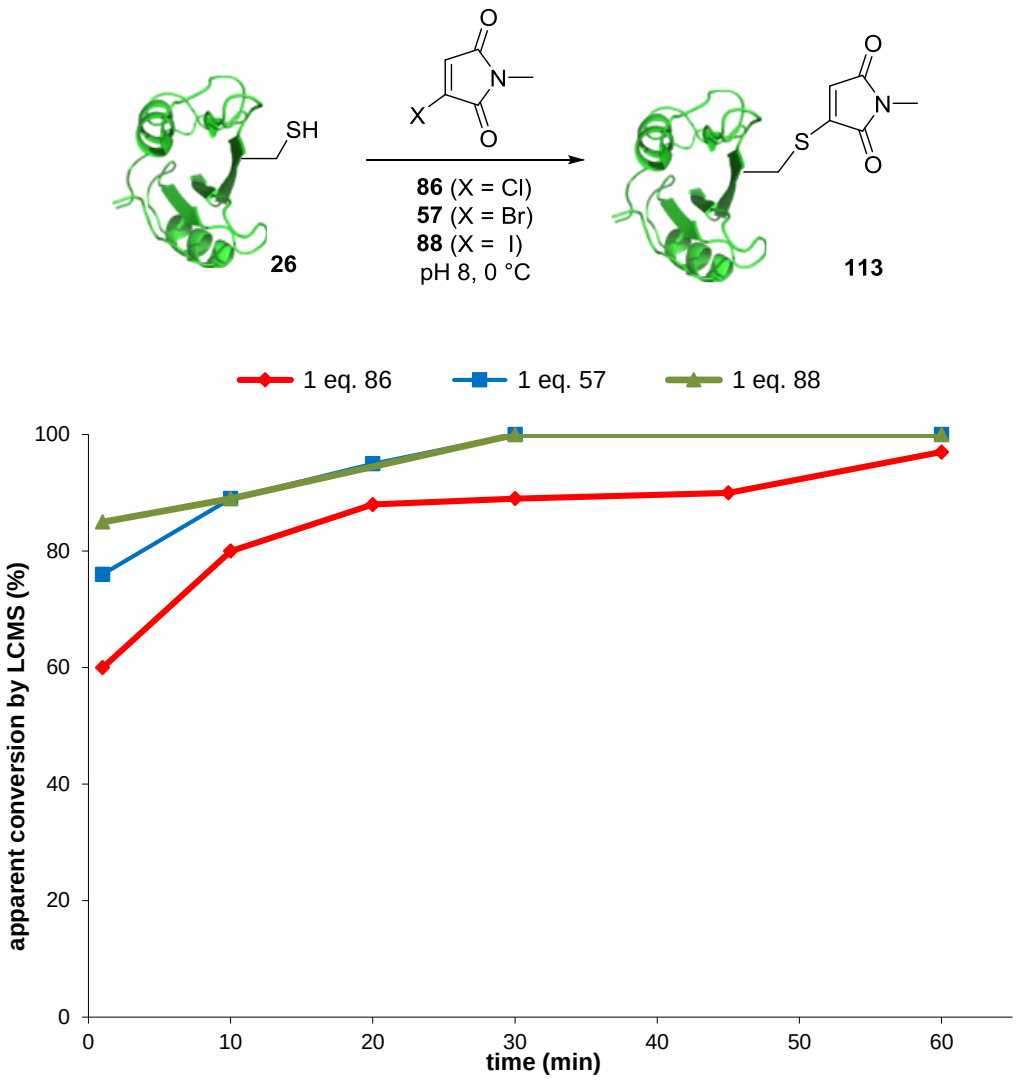


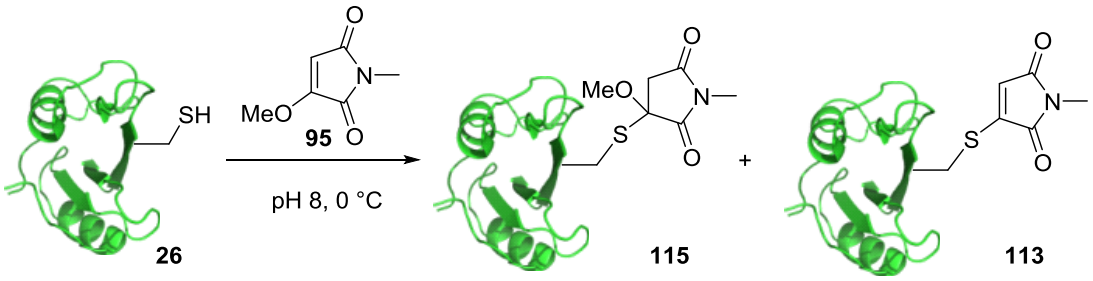
Fig. 16. Labelling of Grb2 SH2 (L111C) with halomaleimides **86, 57** and **88**

In conclusion, the halomaleimides described above are efficient cysteine labelling reagents.

II.2.4 Reactivity of alkoxy- and phenoxymaleimides towards Grb2 SH2 (L111C)

Compound **95** was designed as a representative of an alkoxy-substituted maleimide and compounds **101, 103** and **104** as examples of aryloxymaleimides. To the best of our knowledge, the use of such reagents in the area of protein modification has not been yet reported. The alkoxy and aryloxy substituents have an electron withdrawing effect by induction, due to the high electronegativity of oxygen and an electron donating effect by conjugation, enabled by the lone pair of electrons on the oxygen atom next to the double bond of the maleimide ring. The strong mesomeric effect is expected to activate the α-position to the substituent towards nucleophilic attack by increasing the density of electrons in the β-position.

Treatment of Grb2 SH2 (L111C) **26** with 100 eq. of methoxymaleimide **95** led, after 2 h, to a mixture of Grb2-*N*-methyl thiosuccinimide conjugate **115** (M = 14312), (84%) and Grb2-*N*-methyl thiomaleimide conjugate **113** (M = 14280), (16%).



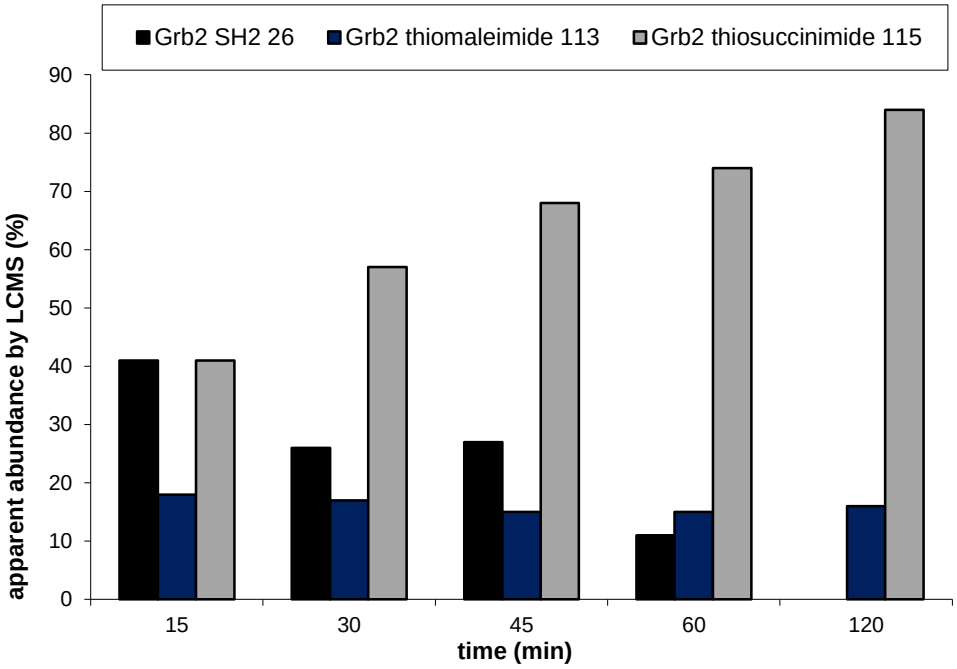
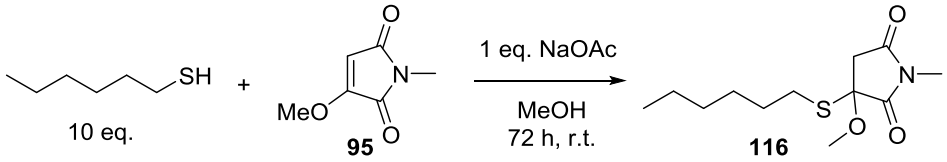


Fig. 17. Reaction of Grb2 SH2 (L111C) with methoxymaleimide **95**

Due to the strong mesomeric effect of the methoxy group, the thiol attack is expected to be α to the oxygen leading to the succinimide conjugate **115** (M = 14312) as drawn in Fig. 17. The high percentage is due to the poor leaving group ability of the methoxy group. The need for such a large amount of reagent and the long reagent times highlight the fact that the alkoxymaleimides are very unreactive compared to the halomaleimides. This is correlated to the strong electron donating effect of the alkoxy group.

To further investigate the regioselectiviy of the thiol addition to methoxymaleimide **95,** it was decided to use *n*-hexane thiol as a model thiol as this would allow ^{1}H-NMR analysis of the product. When 10 eq. of *n*-hexanethiol were reacted with methoxymaleimide **95**, product **116** was obtained almost quantitatively (90%) proving that the thiol attack took place α to the oxygen (Scheme 71). This is to be expected based on the strong mesomeric and inductive effects of exerted by the methoxy group and supports the structure that was proposed for the protein conjugate **115**.



Scheme 71. Reaction between *n*-hexanethiol and methoxymaleimide **95**

As seen in Fig. 18, phenoxymaleimides are significantly less reactive than bromomaleimide **57**. Reaction of Grb2 SH2 (L111C) **26** with 1 eq. of each of the various analogues results in moderate conversions (30% - 60%) after 1 h. All reactions led to the formation of the expected product, Grb2-*N*-methyl thiomaleimide conjugate **113** (M = 14280) only. The difference in the reactivity between **101** and **103** and **104** respectively indicates that fine tuning of the reactivity of these compounds is possible by installing substituents on the phenyl ring. Reagents bearing electron withdrawing group on the phenyl ring are expected to be more reactive than the unsubstituted phenoxymaleimide **101**. Indeed, the experimental results indicate this trend with the nitro substituted phenoxymaleimide **103** being faster than **101**. There is not much difference between compounds **104** and **101**, suggesting that the methyl ester does not exert a strong enough electron withdrawing effect.

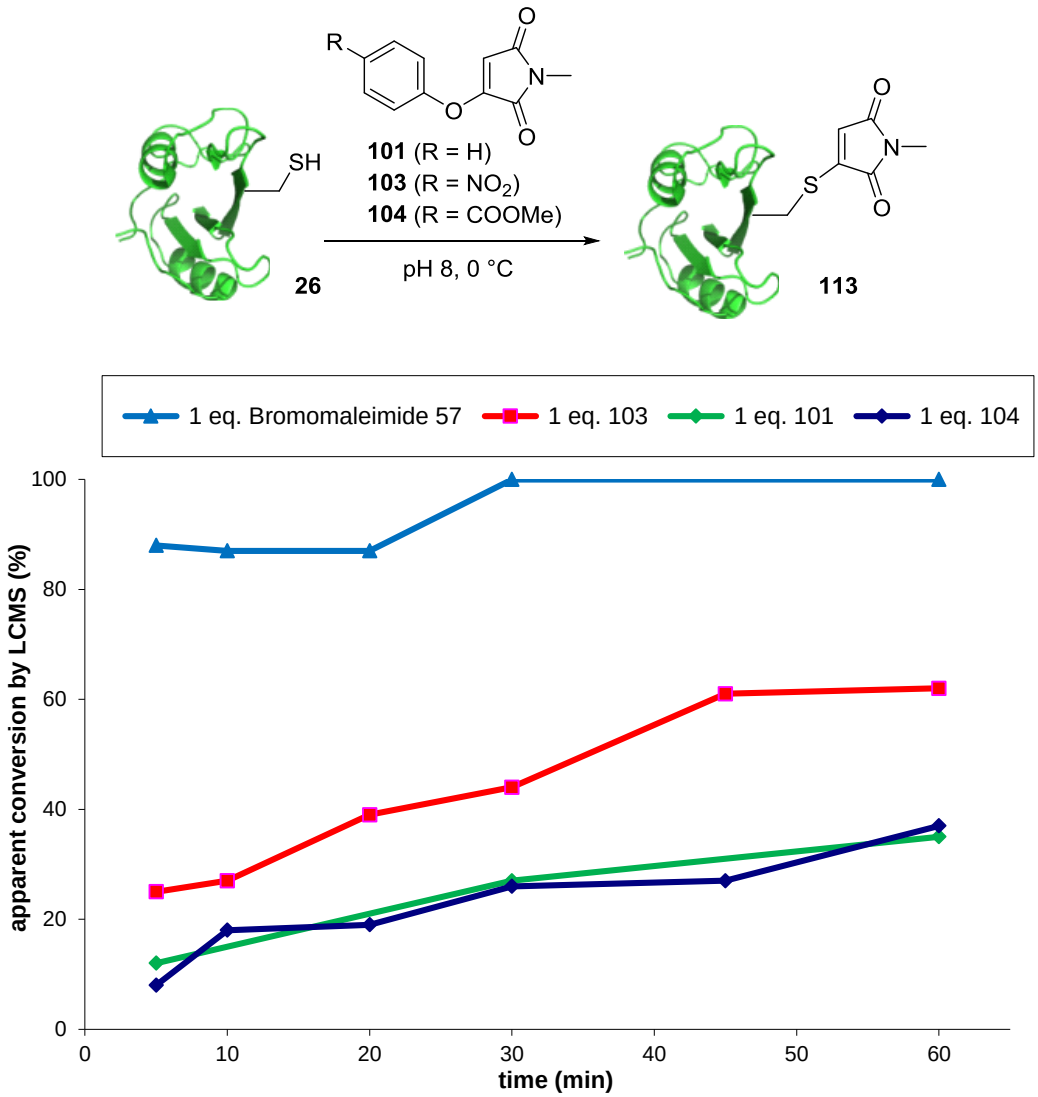


Fig. 18. Reactivity of selected phenoxymaleimides towards Grb2 (L11C) SH2

The reaction between maleimide **103** and Grb2 SH2 (L111C) **26** was monitored over 2 h with a maximum yield of 74% being reached. The rate of reaction was significantly decreased, which could indicate a certain degree of degradation of the reagent or that the reagent was not present in stoichiometric amount. In an attempt to improve the yield of the transformation, the amount of phenoxymaleimide **101** was increased to 10 eq.. Gratifyingly, the reaction reached completion within 10 min.

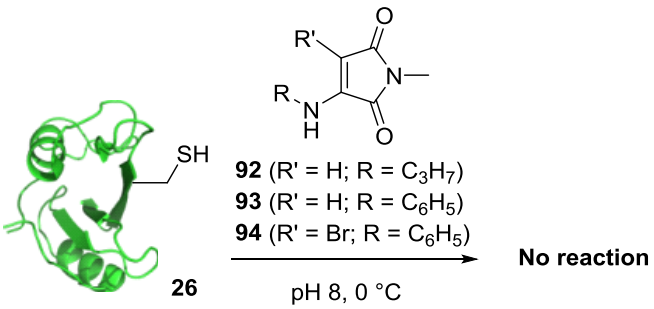
In conclusion, alkoxymaleimides and phenoxymaleimides are less reactive than bromomaleimides. They have a different reactivity profile arising from the electronic effects of the substituents and the leaving group ability difference (pK_a (PhOH) = 10 while pK_a (MeOH) = 15). Alkoxymaleimides lead mainly to addition products such as Grb2-alkoxysuccinimide adduct **115** (M = 14312) while

phenoxymaleimides undergo the addition-elimination sequence giving Grb2-thiomaleimide conjugate **113** (M = 12280) only. Thiosuccinimide type products, similar to the ones obtained when maleimide is added to a thiol, are more resistant in thiol rich envirnments therefore their use is preffered if the conjugate is subjected to such conditions. The use of the less reactive phenoxymaleimide can be of use in the context of selective labelling of the most reactive cysteine only, on a protein substrate bearing at least two cysteines, with enough difference in reactivity. Such differences in the reactivity of cysteine residues have been reported in the literature by Moody et al.[194] and are the basis for dual modification of proteins strategies. Fast and efficient conversion to the maleimide labelled Grb2 SH2 (L111C) **26** was achieved when using a excess (10 eq.) of phenoxymaleimide **101**.

II.2.5 Reactivity of aminomaleimides towards Grb2 SH2 (L111C)

The amino group has a strong electron donating effect by mesomerism which deactivates the maleimide ring towards nucleophilic attack. We were interested to check the degree of deactivation, therefore the reactivity of aminomaleimides **92** - **94** towards cysteine in Grb2 SH2 (L111C) **26** was investigated.

Treatment of Grb2 SH2 (L111C) **26** with 100 eq. of **92, 93** or **94** did not lead to any reaction after 2 h at 0 °C suggesting that, under these conditions, the reagents are completely unreactive towards cysteine (Scheme 72). In conclusion, aminomaleimides are unsuitable for cysteine labelling. This is due to the strong deactivation induced by the amino substituents.

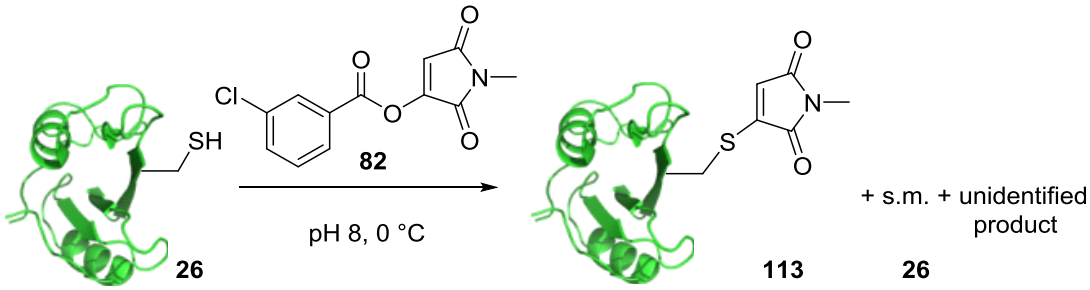


Scheme 72. Reaction of aminomaleimides **92** - **94** with Grb2 SH2 (L111C)

II.2.6 Reactivity of benzoatemaleimide 82 towards Grb2 SH2 (L111C)

As opposed to amino groups, the benzoate group has electron an withdrawing effect both by induction and mesomerism. This is expected to activate the ring towards nucleophilic attack.

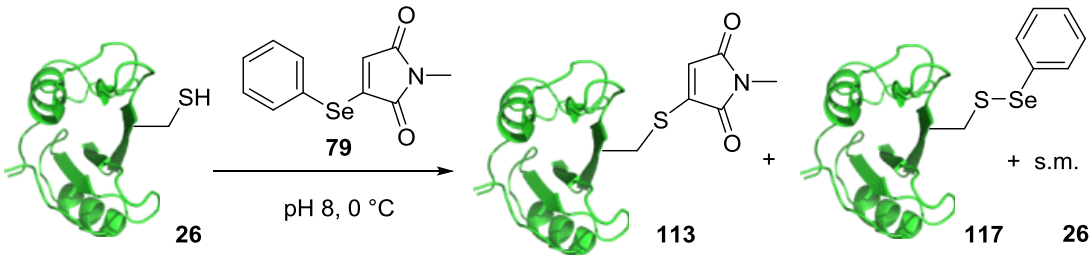
The reaction between 1 eq. of benzoatemaleimides **82** and Grb2 SH2 (L111C) **26** was performed and monitored by LC-MS over 2 h. Scheme 73 shows the composition of the reaction mixture after 2 h: 61% of unreacted Grb2 SH2 (L111C) **26**, 12% of *N*-methyl Grb2 thiomaleimide conjugate **113** and 27% of an unidentified product with a mass of M = 14307. Benzoate maleimide is therefore unsuitable for cysteine labelling as it leads to the formation of a side-product. The conversion could be increased by using excess reagent but that might increase the yield of the side product as well. Further investigation is required.



Scheme 73. Reaction of Grb2 SH2 (L111C) with benzoatemaleimide **82**

II.2.7 Reactivity of selenomaleimide 79 towards Grb2 SH2 (L111C)

Similar with the work with the other reagents, the reaction between Grb2 SH2 (L111C) and 1 eq. selenomaleimide **79** was performed and then monitored by LC-MS over 1 h. The reaction mixture after 1 h contained 20% unreacted Grb2 SH2 (L111C) **26**, 54% the expected Grb2-*N*-methyl thiomaleimide conjugate **113** and 26% of product **117** with a mass M = 14321, for which we suggest the structure shown below (Scheme 74). The formation of compounds such as **117** is documented in the literature and is due to the strong oxidising capacity of the selenide ion. This preliminary result shows the low potential of using this reagent for the cysteine labelling.

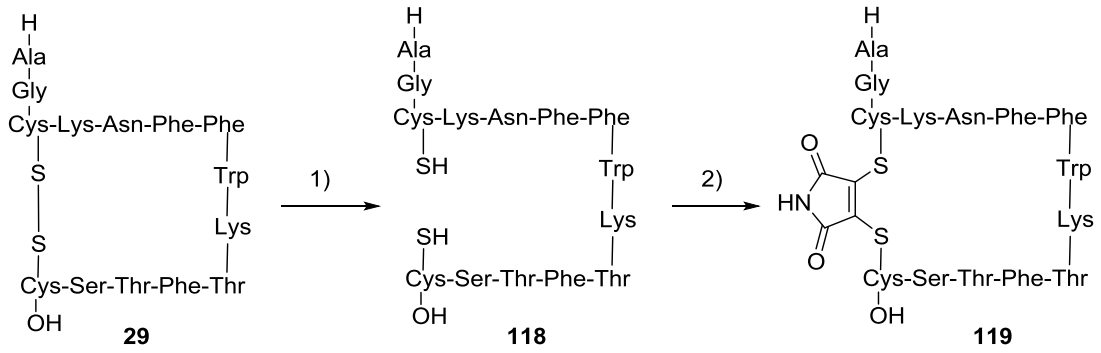


Scheme 74. Labelling of Grb2 SH2 (L111C) with selenomaleimide **79**

II.2.8 Reactivity of thiosulfoxymaleimides towards somatostatin

Having shown that the dithiomaleimides such as **63** can be efficiently employed for disulphide bridging, it was envisaged that the replacement of one of the thiophenol substituents with the more electron withdrawing sulfoxy group would increase the rate of reaction with reduced disulphides whilst preserving the bridging ability of the reagent. In order to assess the utility of this class of reagents, disubstituted maleimides **68** and **69** were reacted with somatostatin **29**, the previously employed model system in the Baker group.

The first step of the stepwise bridging protocol involves the reduction of the disulphide bond in somatostatin. This is achieved by treating somatostatin **29** with 1.5 eq. of TCEP at pH 6.4 (50 mM phosphate buffer, 20% CH_3CN, 2.5 % DMF) for 1 h. In order to probe the completion of the reduction of the disulphide bond, an aliquot of the reduced peptide solution **118** is reacted with with 5 eq. of the commercially available dibromomaleimide **25** for 5 min (Scheme 75).



1) 1.5 eq. TCEP, pH 6, 40% CH_3CN, 2,5% DMF, 1 h, r.t
2) 10 eq. dibromomaleimide **25**, 5 min, r.t.

Scheme 75. Reduction and reduction-probing steps

As the LC-MS spectrum of this mixture showed only the bridged somatostatin **119** (M = 1734), confirming completion of the reduction step, the addition of 1 eq. of disubstituted maleimide **68** was performed (Fig. 19). LC-MS analysis of the reaction mixture after 5 min shows that the mixture consisted of unreacted somatostatin **29** (M = 1640) and bridged product **120** (M = 1748) in a 1 : 2 molar ratio. Approximately the same ratio was obtained when 2 eq. of **68** were employed. It is also important to highlight that the reactions did not proceed any further after 5 min, which is presumably due to the instability of these reagents in water, in a similar fashion to sulfoxymaleimides **66** and **67**. When 6 eq. of maleimide **68** were used, conversion was 90%, proving the potential utility of these reagents to act as disulphide bridging reagents.

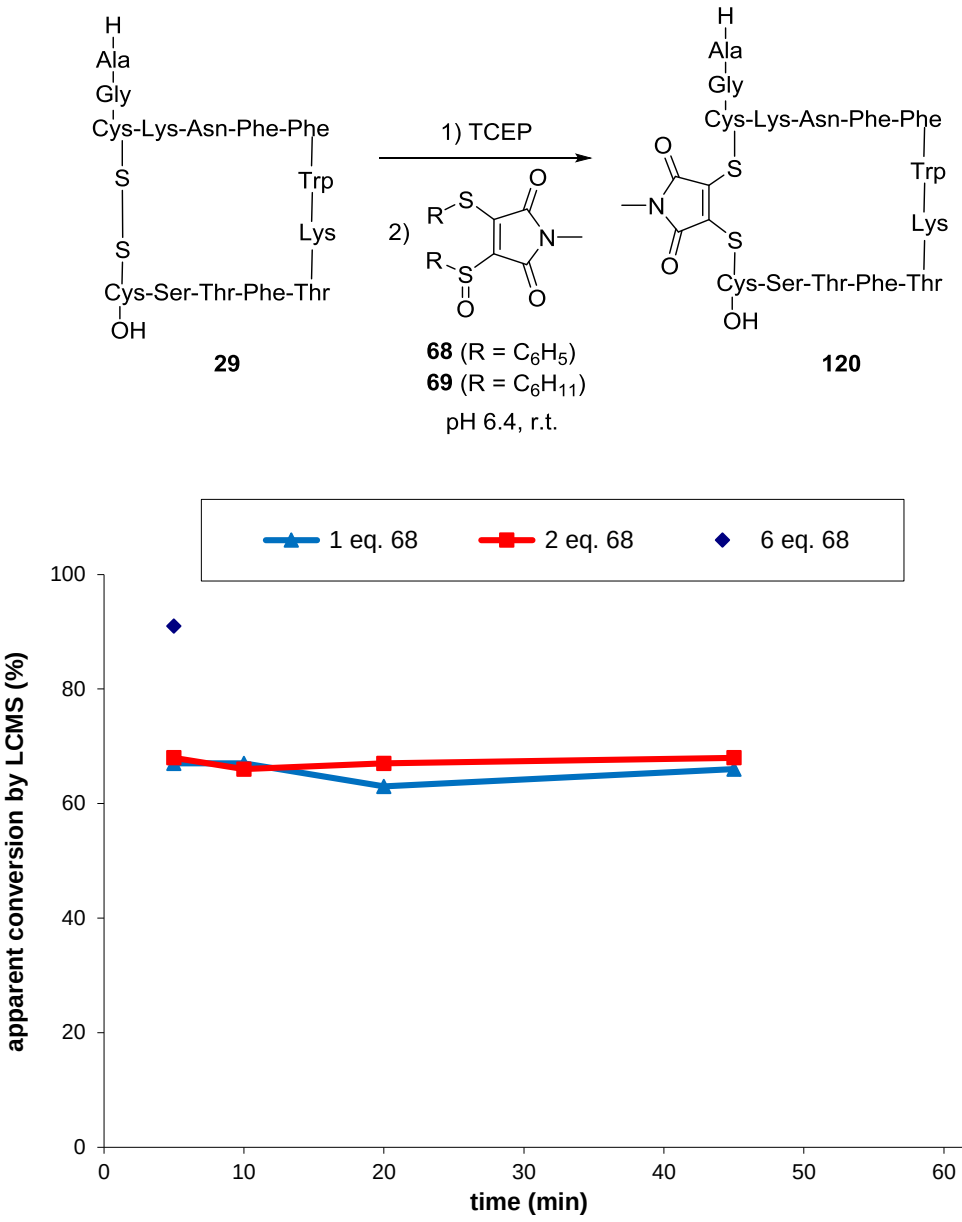


Fig. 19. Stepwise bridging of somatostatin using thiosulfoxymaleimides **68** - **69**

In conclusion, except for some very small peaks, bridging reaction of somatostatin with 6 eq. of maleimide **68** is sufficiently clean. On the other hand, reaction between reagent **69** and somatostatin **29** led to a complex mixture consisting of the expected bridged product (M = 1648) and three other unidentified side products (M = 1756, M = 1982 and M = 2090). In this case, the reduced peptide was fully used up within the first 5 min. This reactivity pattern makes compound **15** unsuitable for disulphide bridging.

II.2.9 Reactivity of diphenoxymaleimides towards somatostatin

The reaction between 1 eq. of diphenoxymaleimide **100** and somatostatin **29** was investigated (Fig. 20). Gratifyingly, the reaction lead to the maleimide bridged somatostatin conjugate **120** (M = 1748). However, the conversion after 1 h was only 34%, much lower than with dibromomaleimide **65**, which gave full conversion in just 10 min with the same number of equivalents.[83] To speed up the reaction, the number of eq. of diphenoxymaleimide **100** was increased to 10 and 100 respectively in which cases the reaction reached completion within 1 h and 10 min respectively.

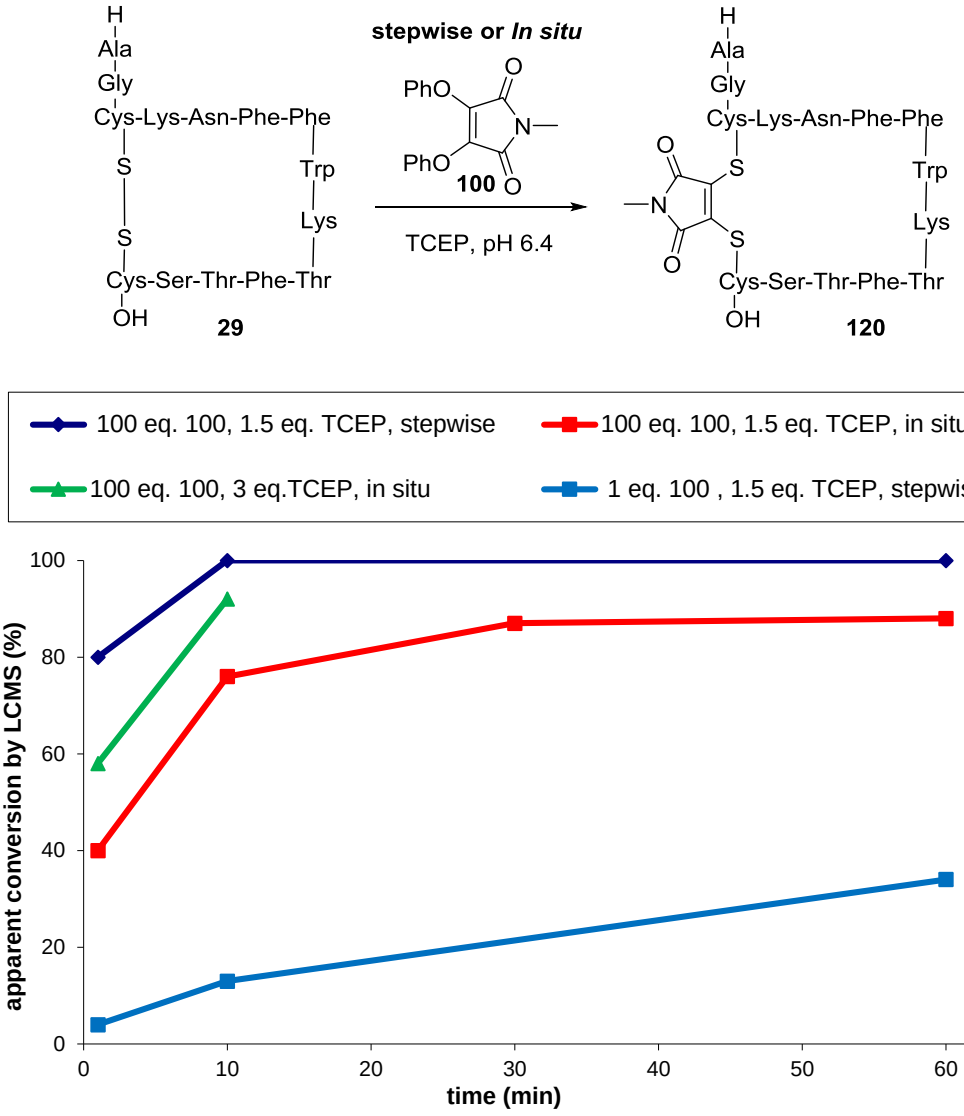


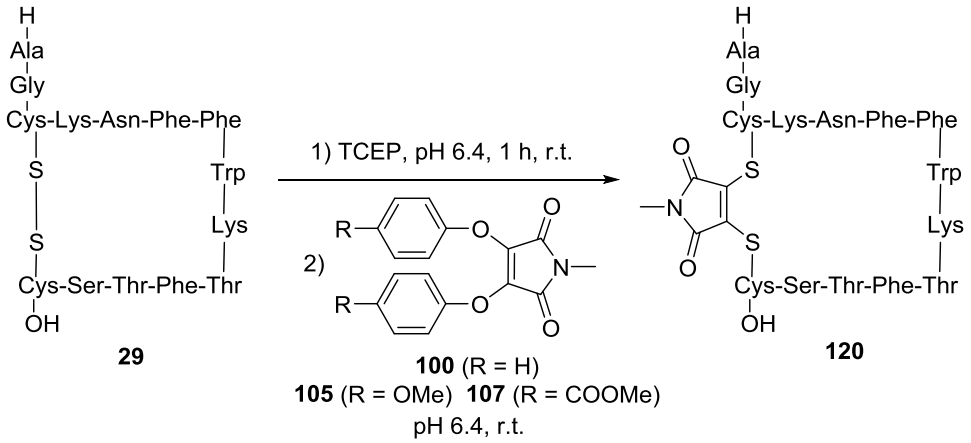
Fig. 20. Bridging of somatostatin with diphenoxymaleimide **100**

Reagent **100** was shown to be compatible with the *in situ* protocol, which consists in adding the reducing agent immediately after the labelling reagent had been mixed with the peptide, so that the disulphide bond reacts as soon as it is

reduced. With 3 eq. of TCEP and 100 eq. of diphenoxymaleimide **100**, the conversion reached after just 10 min was 92 %.

The assumption that reduced somatostatin **118** undergoes oxidation, preventing the reaction from reaching completion is supported by the fact that the conversion increased when the amount of TCEP was increased from 1.5 to 3 eq (Fig. 20). This also suggests that there is no significant cross-reactivity between **100** and the reducing agent TCEP.

It was hypothesised that the reactivity and water solubility of phenoxymaleimides can be tuned by adding substituents on the phenyl rings.



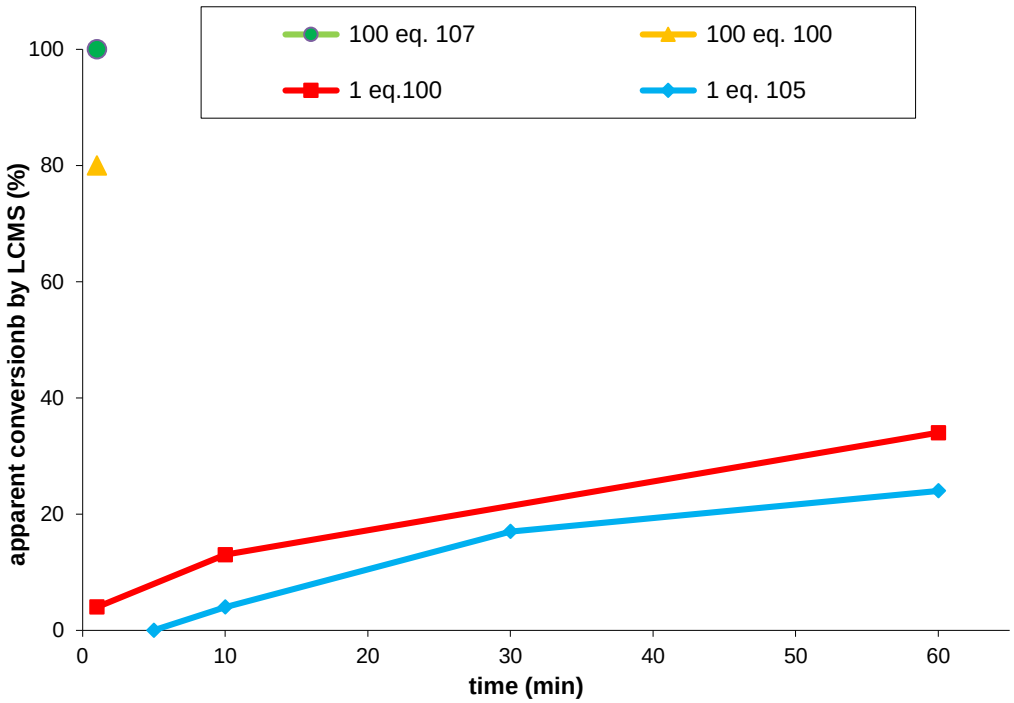


Fig. 21. Reactivity profile towards somatostatin of diphenoxymaleimide **100** compared to analogues **105** and **107**

Electron withdrawing substituents (COOMe, NO_2) are expected to speed up the bridging as they would activate the double bond towards nucleophilic attack, while electron donating substituents (OMe) are expected to have the opposite effect. To illustrate this, di(para-methoxy)phenoxymaleimide **105** (Fig. 21),and, at the other end of the spectrum, di(para-methylester)phenoxymaleimide **107** were synthesised. Indeed, experimental results confirm the expected reactivity trend (Fig. 21).

These results prove that the diphenoxymaleimides are slower bridging reagents compared to dibromomaleimide. The reason for this is the electron donating effect of the phenoxy groups deactivates the double bond towards nucleophlilic attack by increasing the electron charge density. This makes these reagents potentially useful for the selective labelling of the most reactive disulphide bond residues in proteins. They are especially recommended in cases where the *in situ* protocol is preferred, as results indicate no cross-reactivity with TCEP.

II.2.10 Reactivity of halophenoxymaleimides towards somatostatin and Grb2 SH2 (L111C)

This class of compounds is interesting due to their potential to act as disulphide bond labelling reagents but also to illustrate the influence of the substituents on the double bond on the regioselectivity of the thiol attack.

To investigate the regioselectivity of the thiol addition to substituted maeimides, Grb2 SH2 (L111C) **26** was treated with 1 eq. of **99** and the reaction was monitored by LC-MS over 45 min. After this time, the reaction mixture contained unreacted Grb2 SH2 (L111C) **26** (70%), the rest being a species with M = 14359 that corresponds to structure **121** (Fig. 22). The formation of a sole protein conjugate suggests a clear selectivity for phenoxy displacement over bromine.

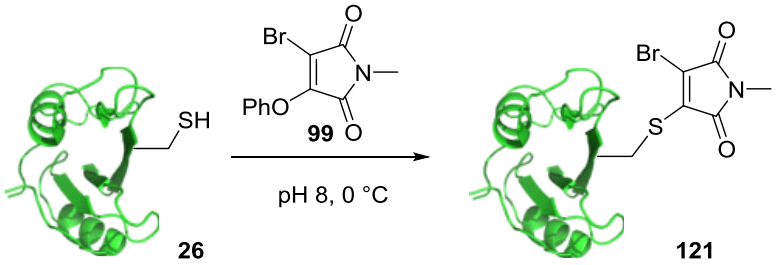
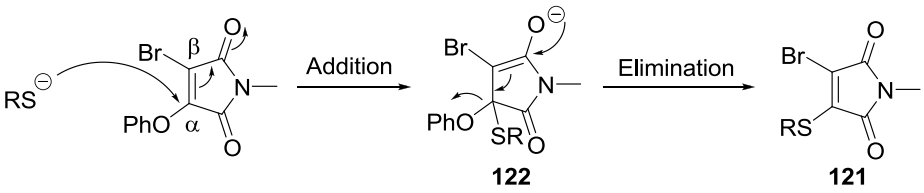


Fig. 22. Reaction of Grb2 SH2 (L111C) with halophenoxymaleimide **99**

The proposed mechanism is as follows: firstly, the thiolate attacks the α-position on the double bond, which is the carbon with the lowest electron density to give intermediate **122**; this is followed by elimination of the phenoxy group, to give

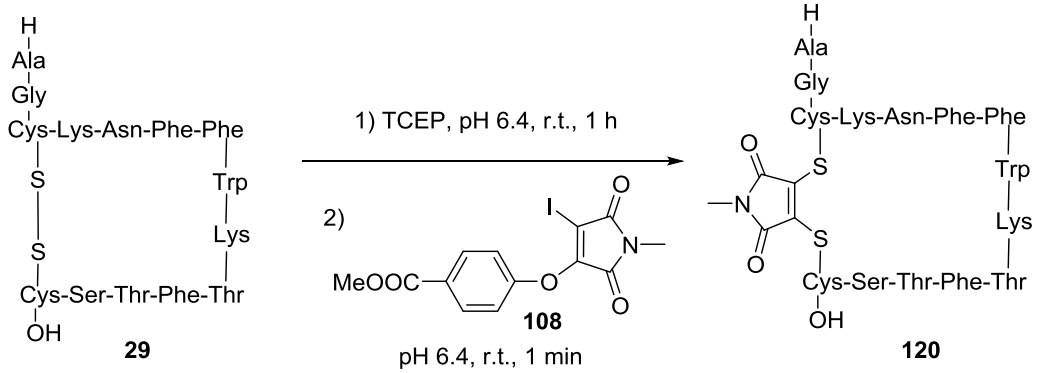
product **121** (Scheme 76). This shows that it is the electronic effect of the leaving groups and not the leaving group ability (pK_a) that dictates which of the two substituents is displaced by thiol.



RSH = Grb2 SH2 (L111C) **26**

Scheme 76. Postulated mechanism for the formation of conjugate **121**

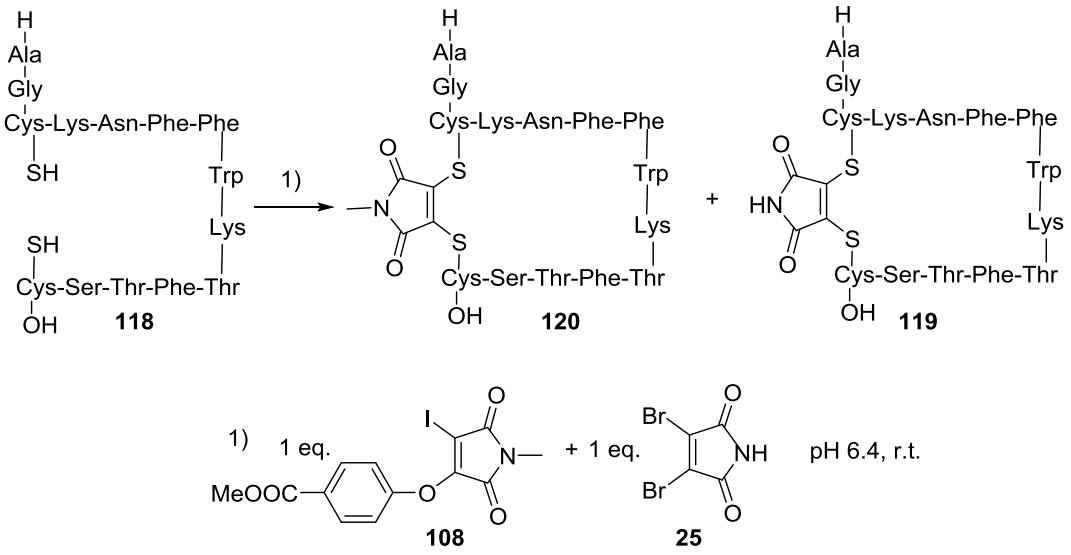
Next, the potential utility of these reagents to act as disulphide bridging reagents was explored. Using the stepwise protocol reaction conditions, the labelling of somatostatin **29** with 1 eq. iodophenoxymaleimide **108** was complete within 1 min, which makes it the fastest disubstituted maleimide tested so far (Scheme 77). Reagent **108** performed less well in the *in situ* protocol (1.5 eq. of TCEP, 1 eq. iodophenoxymaleimide **108**) where the conversion was 73%, after 40 min, which could indicate a certain degree of cross-reactivity of such reagents with TCEP.



Scheme 77. Stepwise bridging of somatostatin with iodophenoxymaleimide **108**

To further asses the reactivity of these reagents, a competition experiment with dibromomaleimide was designed (Scheme 78). The experiment is based on the fact that the two reagents have a different substituent on the nitrogen atom, giving somatostatin conjugates with different masses. When a mixture of 1 eq. of iodophenoxymaleimide **108** and 1 eq. of dibromomaleimide **25** was treated with 1 eq. of somatostatin **29**, a 40 : 60 mixture of *N*-methyl maleimide bridged somatostatin adduct **120** (M = 1748) and maleimide bridged somatostatin conjugate **119** (M = 1734) was obtained. This shows that reagent **108** is slightly less reactive

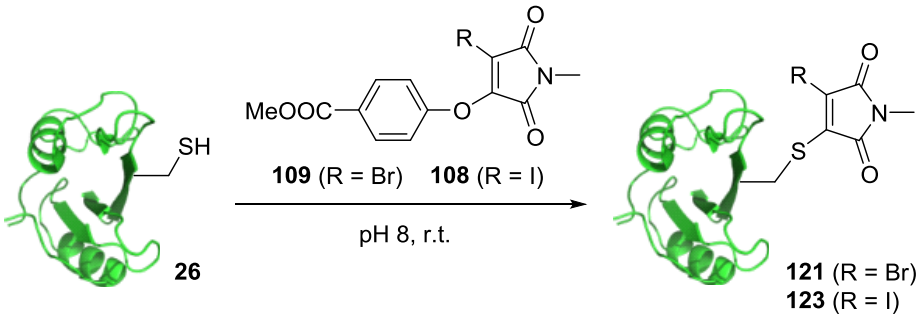
towards somatostatin **29** compared to dibromomaleimide **25**. This feature could become more obvious on a less reactive substrate.



Scheme 78. Competition experiment between reagents **108** and **25** for bridging somatostatin

Reaction of 1 eq. of iodophenoxymaleimide **108** with Grb2 SH2 (L111C) **26** gave conjugate **123** proves that the attack of the thiol takes place α to the oxygen part of the phenoxy group, as expected considering the strong mesomeric effect of the oxygen part of the phenoxy group.

In order to evaluate the contribution of the iodine substituent in the reactivity of **108**, the corresponding bromomaleimide analogue **109** was synthesised and then the reactivity of the two reagents was assessed using Grb2 SH2 (L111C) **26** as model system. Fig. 23 shows that **108** is marginally faster than **109**, which suggests that the iodine has a weaker deactivating effect on the β-carbon atom compared to bromine. This is in accordance with the trend of increasing strength of the mesomeric effect in the order I < Br, which is proportional to the orbital overlap between the orbital of the carbon and the halogen atom.



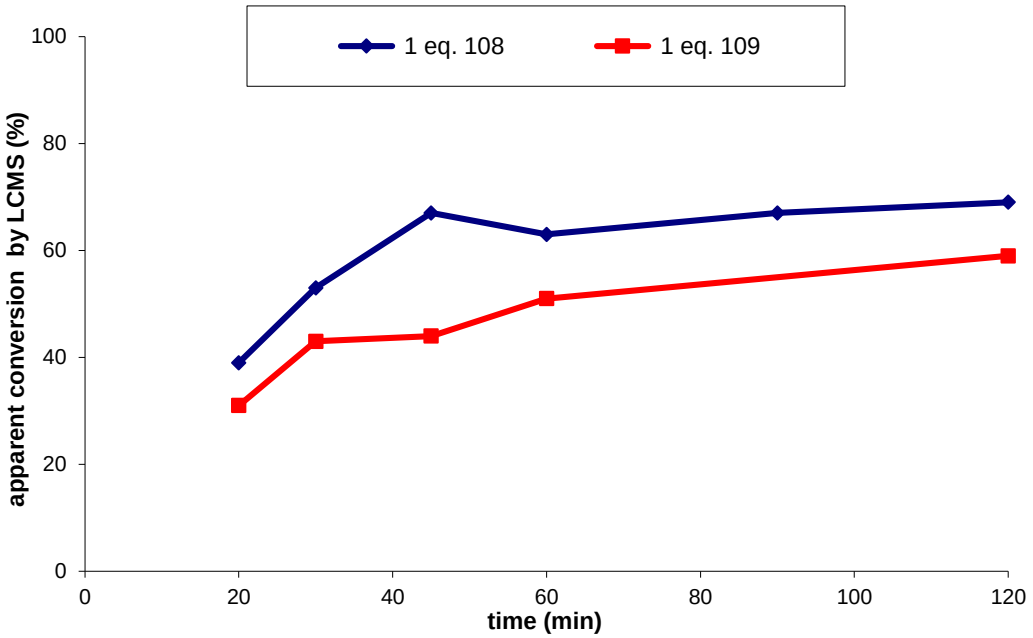
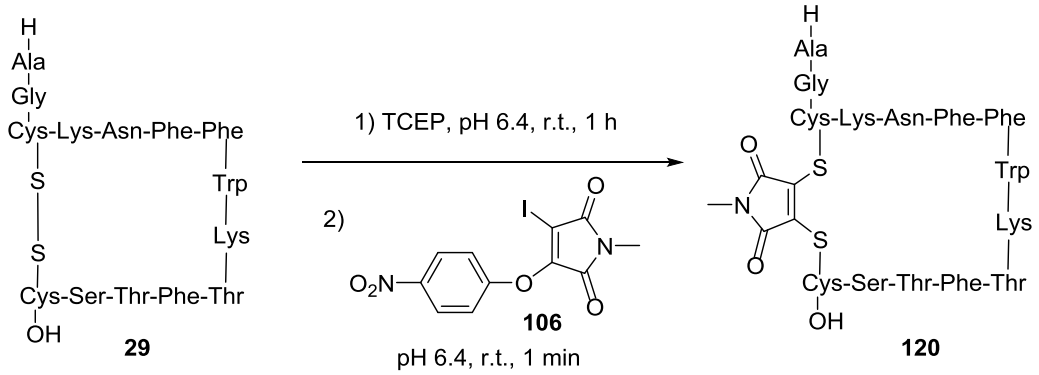


Fig. 23. Comparison between the reactivity of compounds **108** and **109** towards Grb2 SH2 (L111C)

The same experiments were repeated using iodophenoxymaleimide **106**. In the stepwise protocol complete labelling of somatostatin **29** was achieved in 1 min (Scheme 79). Following the *in situ* protocol (1.5 eq. of TCEP, 1 eq. iodophenoxymaleimide **106**) a maximum conversion of 61% was achieved after 55 min. A 93% conversion was achieved by increasing the amount of TCEP to 3 eq.. This supports the assumption that the lower conversions in the experiment carried out with 1.5 eq. TCEP are due to the reoxidation of somatostatin rather than cross-reactivity between the labelling reagent and TCEP.



Scheme 79. Stepwise bridging of somatostatin with iodophenoxymaleimide **106**

In conclusion, halophenoxymaleimides are very fast disulphide bridging reagents, very similar in reactivity to the dibromomaleimide, as suggested by the competition experiments. The conversions are very high both in the *in situ* and

stepwise protocols, indicating a negligible cross-reactivity between the halophenoxymaleimides and TCEP. They are also very useful compounds as they allow the study of the regioselectivity of the thiol addition to the disubstituted maleimide.

II.2.11 Reactivity of bromochloromaleimide 91

The asymmetric dihalomaleimide **91** represents an interesting substrate for the study of the regioselectivity of the thiol addition to substituted maleimides. The reaction between 1 eq. of bromochloromaleimide **91** was performed, under standard conditions and the reaction mixture was analysed by LC-MS over 1 h. Fig. 24 shows the abundance of the products present in the reaction mixture.

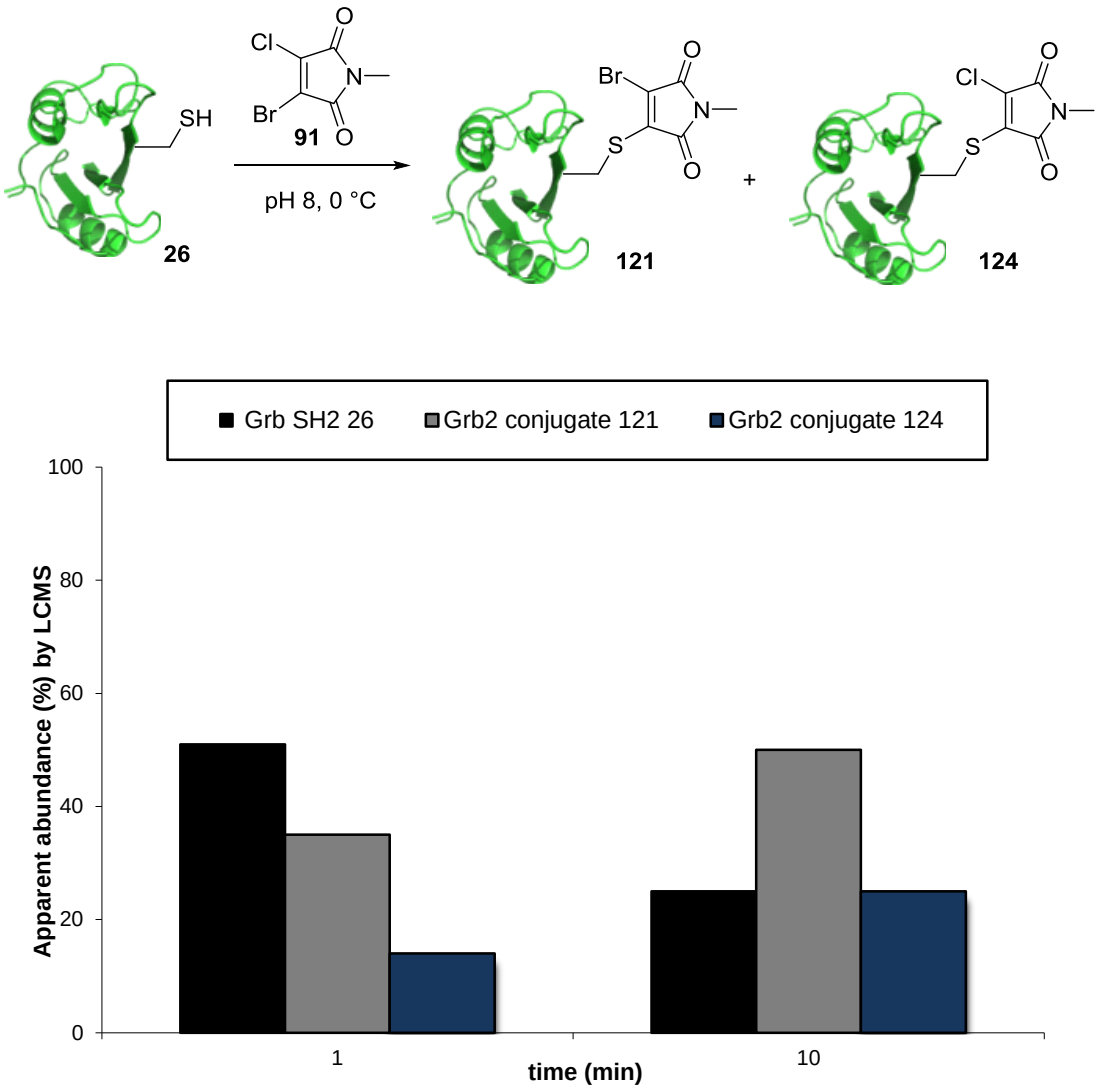


Fig. 24. Labelling of Grb2 SH2 (L111C) with bromochloromaleimide **91**

The first observation that can be made is that the reaction does not reach completion in the time-frame investigated, with around 23% of the unreacted Grb2 SH2 (L111C) protein being detected in the reaction mixture after 1 h. This can be due to an error in the measurement of exactly 1 eq. of reagent. The other observation is that the reaction does lead to two different products, **121** and **124**, indicating that the attack of the thiol takes place at both positions on the double bond.

Additionally, the experimental results indicate that product **121** is formed preferentially over product **124**, the final abundances in the reaction mixture after 10 min being 50% for **121** and 25% for **124**. The reaction was monitored for another 50 min, with no significant improvement in the conversion.

To explain the formation of conjugate **121** as a major product, it is essential to consider the the electronic effects of the two substituents: chlorine and bromine are both electron withdrawing by induction (I-) and electron donating by conjugation (M+), (Fig. 25). Both effects activate the α-position to the halogen towards nucleophilic attack. Based on the variation of the strength of these effects as depicted in Fig. 25, it was predicted that the preffered attack will be at the α-carbon atom with respect to the chloro substituent. Formation of product **121** as the major product confirms that it is the electronic effect of the substituents and not the leaving group ability that is key for the regioselectivity of the reaction.

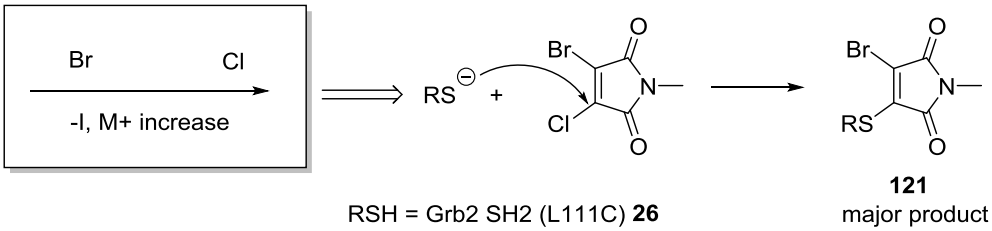


Fig. 25. Electronic effects of bromine and chlorine and the thiol addition to bromochloromaleimide **91**

II.3 Kinetic study

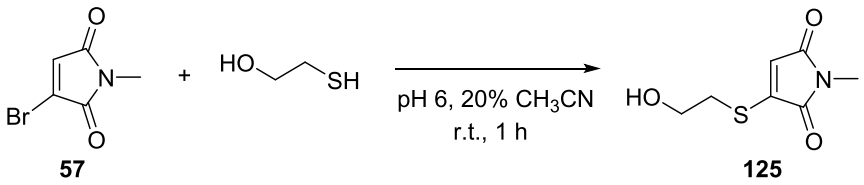
II.3.1 Purpose

The kinetic study was performed with the aim of quantifying and comparing the reactivity of various monosubstituted maleimides towards the free cysteine groups in proteins. So far, the standard method employed by our group has been to monitor the reaction between the maleimide under investigation and Grb2 SH2 (L111C) by LC-MS.[139] This method allows the comparison between different reagents and gives a qualitative indication of reaction rate, but lacks accuracy. Additionally, the monosubstituted halomaleimides (iodomaleimide **88**, bromomaleimide **57** and chloromaleimide **86**) labelled Grb2 SH2 (L111C) very quickly, the only time point where a difference could be detected being after 1 min. The need for an assay with better time resolution became clear. A kinetic study was consequently designed to enable a more quantitative comparison between the reagents tested by finding the rate equation for the addition of cysteine to monosubstituted maleimides.

II.3.2 Selection of the method and preliminary tests

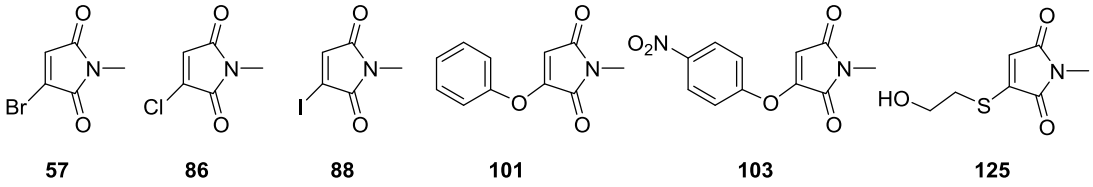
The chosen method to monitor the reaction was UV-spectroscopy. Thiomaleimides show an absorbance maximum around 350 nm, therefore it was decided to see whether this could be exploited.[177] It was envisaged that the reaction progress could be monitored by the increase in the absorbance of the peak corresponding to the thiomaleimide product. For this to be possible, the two starting materials (the maleimide and the thiol) must not absorb in the same region as the product and the reaction must yield thiomaleimide only.

Initial tests were conducted using bromomaleimide **57**, which does not absorb above 300 nm. The UV-spectra of Grb2 SH2 (L111C) **26** was recorded. Unfortunately, the protein showed a significant absorbance in the region of interest, 350 nm. We then focused on finding a small molecule thiol and selected 2-mercaptoethanol, mainly due to its water solubility. As it did not absorb around 350 nm, it became the reagent of choice. The reaction between bromomaleimide **57** and 2-mercaptoethanol was performed in pH 6 buffer, containing 20% CH_3CN to ensure the solubility for all reagents. Gratifyingly the UV-spectrum of the reaction mixture after 1 h, showed a peak with an absorbance maximum at 360 nm, that was attributed to the thiomaleimide product **125** (Scheme 80).



Scheme 80. Reaction of maleimide **57** with 2-mercaptoethanol

As the initial results were promising, it was important to make sure that the other maleimide analogues obeyed the condition stated above; thus the UV-spectra of bromomaleimide **57,** chloromaleimide **86,** iodomaleimide **88**, phenoxymaleimide **101** and 4-nitro phenoxymaleimide **103** were recorded under the reaction conditions. Fig. 26 shows that except for the 4-nitro phenoxymaleimide **103**, the rest of the analogues that we selected for this kinetic study do not present a significant absorbance at 360 nm.



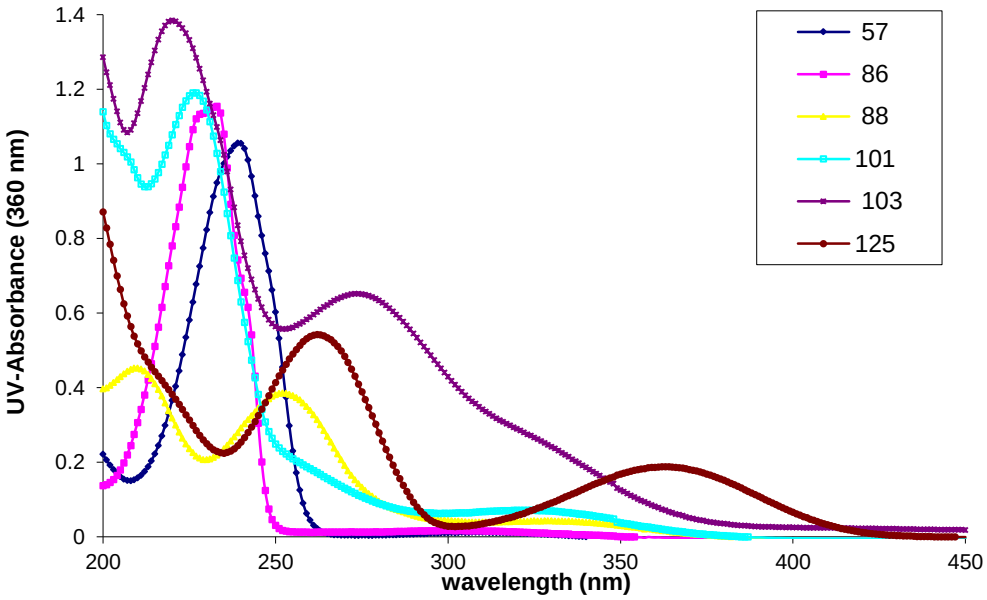


Figure 26. UV-absorbance spectra of substituted maleimides and 2-mercaptoethanol (67 μM in phosphate buffer pH 6, 20% CH_3CN)

II.3.3 Results obtained with 2-mercaptoethanol as model system

Several parameters (pH, temperature, concentration of reagents, setup) were varied in order to determine the optimal reaction conditions. The optimised concentration of the two reagents is 167 µM in phosphate buffer pH 6 (20% CH_3CN). Reactions were done at room temperature.

A calibration curve was plotted using different concentrations of 2-mercaptoethanolmaleimide **125** synthesised in the lab (Fig. 27).

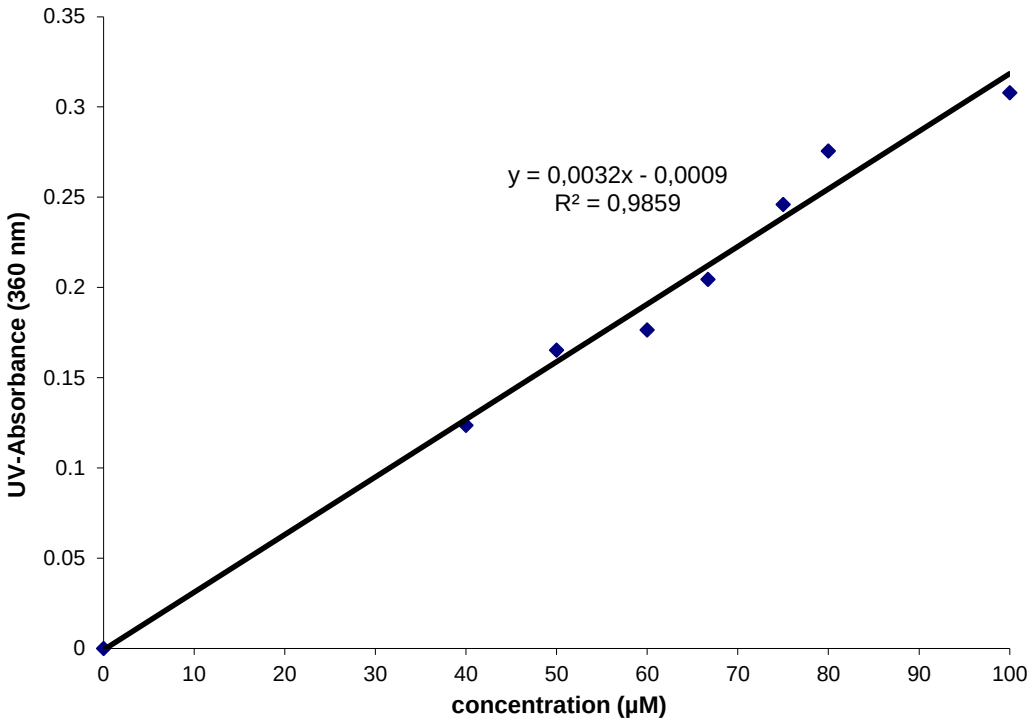


Fig. 27. Calibration curve for mercaptoethanolmaleimide **125** (concentrations between 40 µM and 150 µM in phosphate buffer 20% CH_3CN)

The linear equation shown below will allow the correlation of the absorbance peak at 360 nm (x) to the concentration of thiomaleimide formed (y) and is essential for signalling the end-point of the reaction.

$$y = 0.0032 \cdot x - 0.009 \qquad \text{(Equation 1)}$$

The reaction between **57** and 2-mercaptoethanol was performed under the optimised conditions and the absorbance peak at 360 nm was monitored over 10 min (Fig. 28).

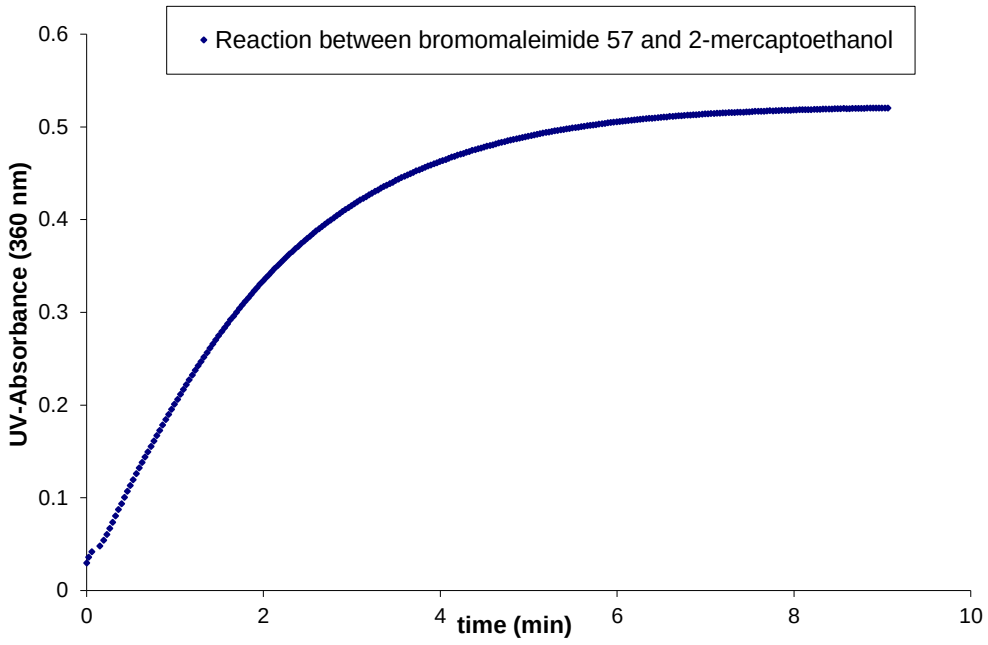


Fig. 28. Experimental data for the reaction of bromomaleimide **57** and 2-mercaptoethanol to the model system

Experimental data was corrected by substracting the signal given by the background from the experimental values of the absorbance:

$A_{corrected}=A_{360} - A_{bk}$, where A_{360} = absorbance of the sample at 360 nm

A_{bk} = absorbance of the solvent at 360 nm

Then the difference between the final (t = 10 min) and the initial (t_0 = 0 min) absorbance at 360 nm, ΔA, was calculated which, was introduced in Equation 1 to generate the corresponding thiomalemide concentration. This corresponds to a conversion of 95%. The next step was fitting for the experimental data with a theoretical model. The rate equations for first and second order reactions were tested but they did not fit the experimental curve. Further attempts to determine the order of the reaction (i.e. the method of pseudo first order conditions) were not possible in our case as the system did not allow use of excess thiol, as this would convert the product into a dithiosuccinimide which could not detected by UV-spectroscopy.

We then tested the other maleimides, under the same conditions: concentration of the two reagents was 167 μM in pH 6 phosphate buffer (20% CH_3CN) and room temperature. The reactions with iodomaleimide **88** and chloromaleimide **86** were monitored over the same period of time, 10 min, whereas

the phenoxymaleimide **101** proved to be a much slower reagent therefore the progress of the reaction was monitored over 60 min (Fig. 29). The reaction curves reached a plateau at the time when the acquisition was stopped so it is assumed that either reached completion or an equilibrium point.

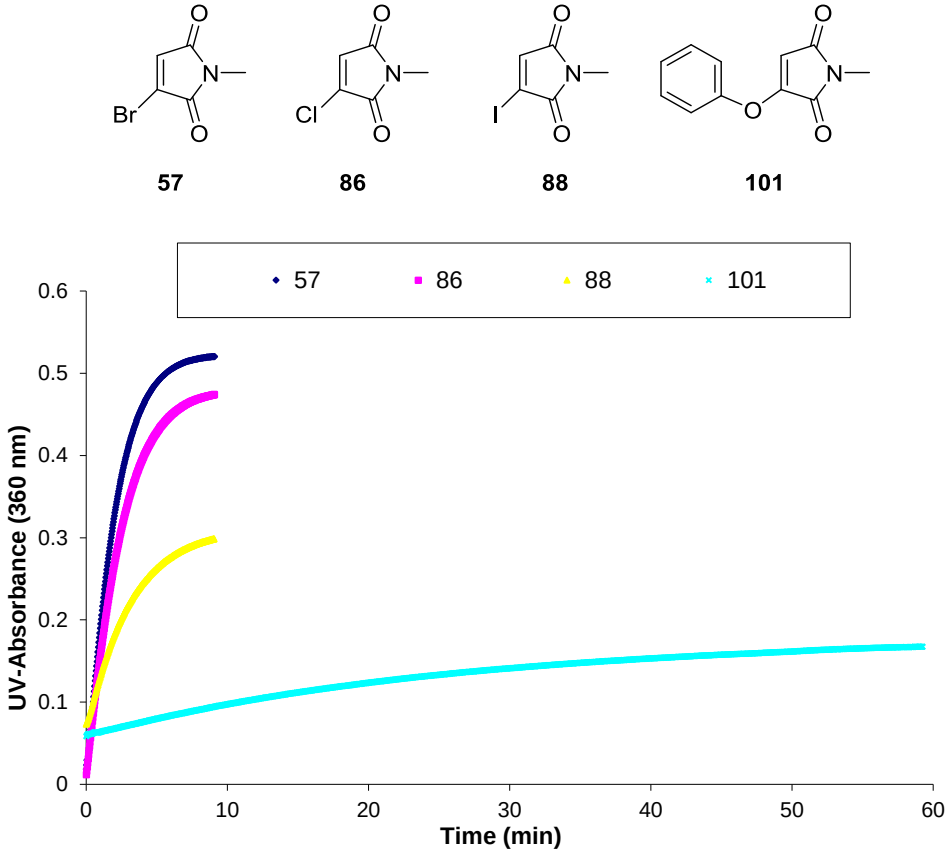


Fig. 29. Comparison between the reaction profiles of monosubstituted maleimide analogues with 2-mercaptoethanol

The same analysis of the experimental data was performed for the other analogues (Table 4).

Maleimide	ΔAbs	Yield thiomaleimide 125 (%)	Δ Abs corrected	Corrected yield thiomaleimide 125 corrected (%)
57	0.507	95	-	-
86	0.469	88	0.462	86
88	0.232	43	0.287	54
101	0.153	31	0.192	36

Table 2. Conversions of the reactions of selected substituted maleimides with 2-mercaptoethanol

The influence of the maleimide on the absorbption at 360 nm was considered and corrected values for the conversion were obtained. The difference is most obvious in the case of iodomaleimide **88** which is the analogue with the highest absorbance at 360 nm.

In conclusion, experimental results show an unexpected feature of these reactions: they do not reach completion. The phenoxymaleimide **101** was expected to be a slow reagent from the way it reacted with Grb2 SH2 (L111C). Results based on UV-absorbance data confirm this but the fact that the yield with respect to the thiomaleimide is low (~30%) and that it does not seem to increase if left to react for longer could be due to the oxidation of the 2-mercaptoethanol or degradation of the labelling reagent *via* hydrolysis.

A more surprising result is the one for the reaction between iodomaleimide **88** and 2-mercaptoethanol, which was expected to be the fastest reaction. However the amount of thiomaleimide **125** formed was very small (~50%), which indicates that some side reactions are taking place. The distinguishing factor for this reaction from the other two involving the bromo- and chloromaleimide respectively is that the iodide is the most nucleophilic. Side reactions could therefore involve addition of the iodide onto the thiomaleimide product, leading to a iodothiosucinimide.

In conclusion, 2-mercaptoethanol is not a suitable model system as the reactions with the different maleimides do not reach completion. This is in direct contrast with the results obtained for the Grb2 SH2 (L111C) **26**, where LC-MS results indicate the reactions have gone to completion. The reasoning that was found for this difference in reactivity is that 2-mercaptorethanol is a small molecule and therefore it is more reactive (i.e. due to steric factors, electronic effects) compared to a protein such as Grb2 SH2 (L111C) **26**. A new model system was required and after a screening of several thiol containing molecules, reduced gluthathione became the reagent of choice as it showed no absorbtion around 360 nm and was readily available.

II.3.4 Results obtained with gluthathione as model system

After an initial screening of optimal conditions (pH, concentration, amount of organic solvent), 75 µL of reduced GSH (300 µM in pH 6 50 mM phosphate buffer) were added to 75 µL bromomaleimide **57** (300 µM in 50 mM phosphate buffer pH 6, 20% CH_3CN) and the reaction mixture was left to stand for 1 h at room temperature. Fig. 30 shows the UV spectra of the starting material and the reaction mixture after 1

h. A clearly visible peak with a maximum at 360 nm was observed, alongside the disappearance of the peaks characteristic to the starting materials.

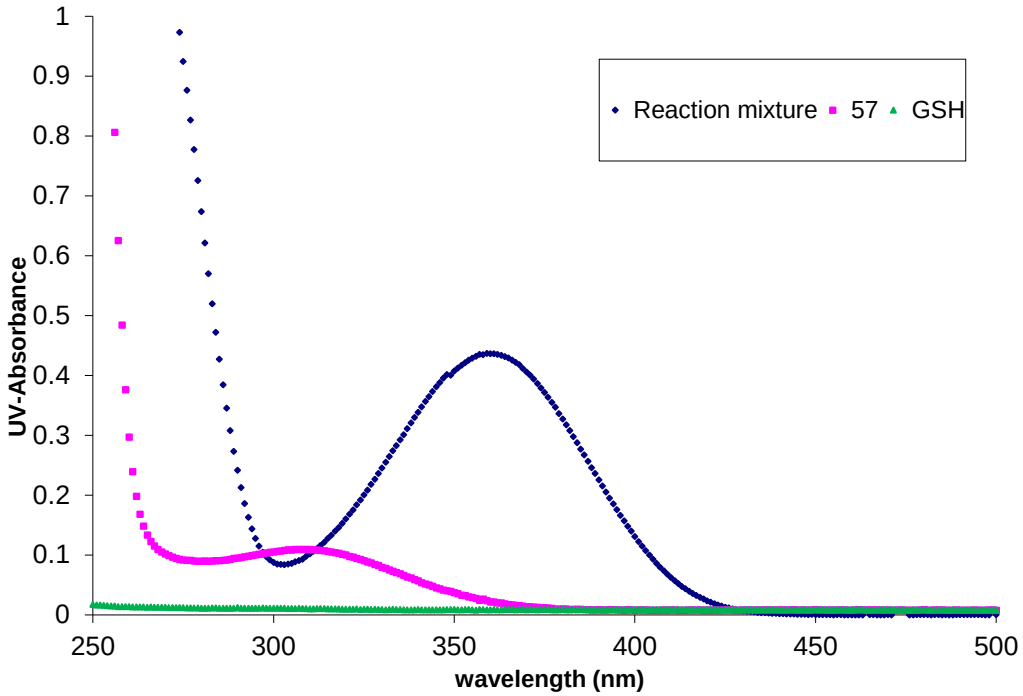
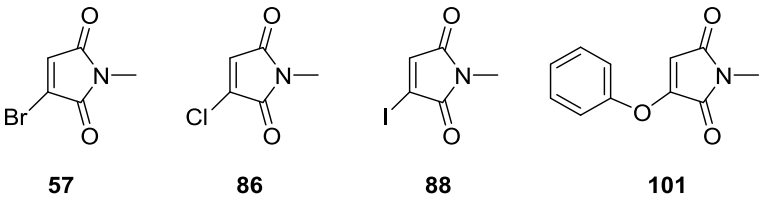


Fig. 30. UV absorbance spectra of the starting materials and of the reaction mixture of **57** (300 μM) with GSH (300 μM) in phosphate buffer pH 6 (10% CH_3CN) after 1 h and background correction

As with the experiments with 2-mercaptoethanol, the yield of the thiomaleimide product was corrected after recording the UV spectra of the maleimide partner. Next, the reactions between GSH and the three halomaleimides were performed and monitored by the evolution of the peak at 360 nm. As can be seen from Fig. 31, the reaction progresses up to a maximum value of the absorbance and then instead of the expected plateau, is followed by a descending curve. This tendency is most significant for the iodo derivative **88**. Same as before, the explanation for the disappearance of the product is attributed to the side reactions promoted by the iodide on the product (e.g. addition of the halide ion to the resulting thiomaleimide). The yields for all three of the reactions were calculated and are given in Table 3. A corrected version of the yields, that considers the influence of the maleimides on the product peak, is also provided.



57 **86** **88** **101**

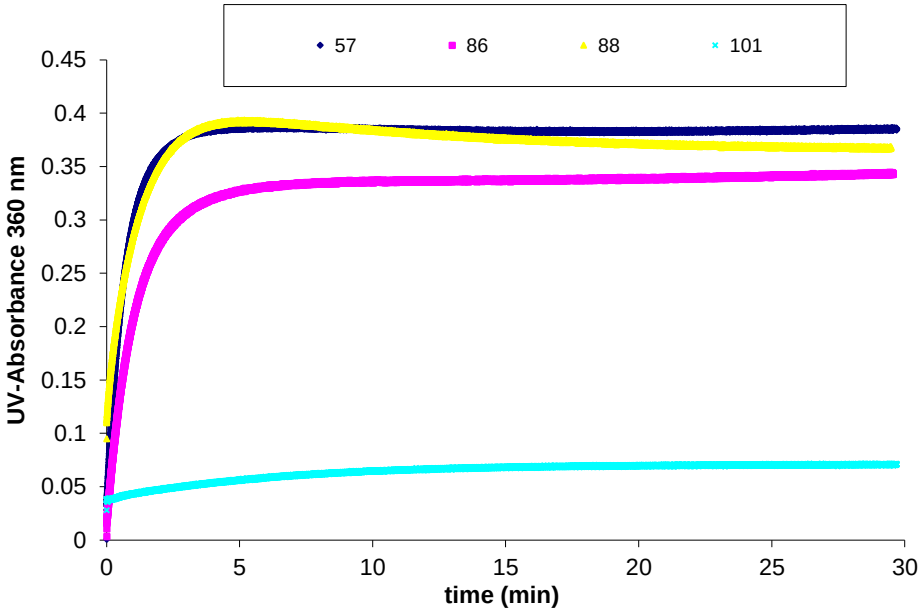


Fig. 31. Reaction profiles between GSH and the various maleimide analogues at 150 μM in phosphate buffer pH 6 10% CH_3CN

From this moment on, the analysis of data was based on the assumption that at the time point where the peak at 360 nm was at its maximum, the reaction reached completion. The reaction between bromomaleimide **57** and GSH was taken as a reference and it was assumed to be quantitative.

Maleimide	ΔAbs	Yield thiomaleimide 126 (%)	ΔAbs corrected	Yield thiomaleimide 126 after correction (%)
57	0.387	100	-	-
86	0.339	87	0.342	89
88	0.298	77	0.392	100

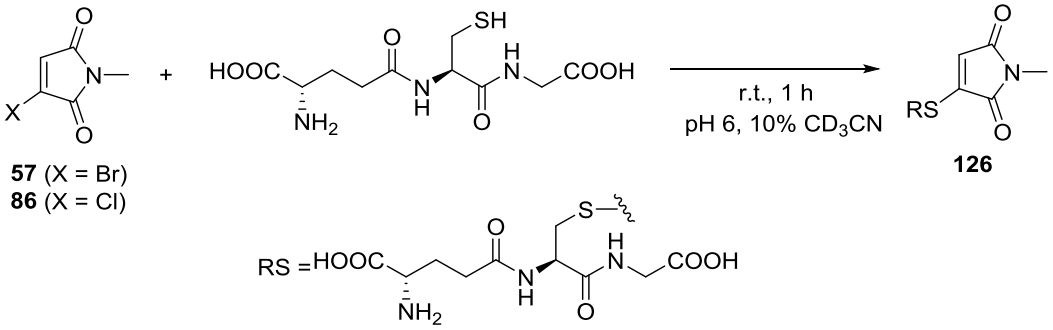
Table 3. Conversions for the reactions between halomaleimides and GSH

Reaction profiles were similar to the ones obtained with 2-mercaptoethanol. Our attempts to fit the experimental data to a simple rate equation were unsuccessful. This suggests that the system is characterised by a complex rate equation. The data obtained is in accordance with the LC-MS data on Grb2 SH2

(L111C) protein: phenoxymaleimide **101** is significantly slower than halomaleimides **57**, **86**, **88** and that chloromaleimide **86** is slower than bromomaleimide **57**.

As mentioned above, the reaction of maleimides with GSH cannot be accurately monitored by LC-MS because of the low molecular weights involved. It was decided to attempt to use ^{1}H-NMR to analyse the reaction mixtures for the chloro- and bromomaleimide and in this way explain the disappearance of the product and why the reaction with chloromaleimide **86** does not reach completion.

The reactions were carried out at a concentration of 1.67 mM in 150 μM phosphate buffer pH 6 containing 10 % CD_3CN. The high concentration was necessary to ensure a good quality ^{1}H-NMR spectrum for product **126**. The samples were prepared by mixing the reaction mixture with deuterium oxide in a 9 : 1 volume ratio the spectrum was obtained on a 400 MHz NMR spectrometer, using a Water suppression tool and having 80 scans. The ^{1}H-NMR spectra for the reactions of the two halomaleimides signalled the glutathionethiomaleimide conjugate **126** based the peak at 6.56 ppm (Scheme 81).



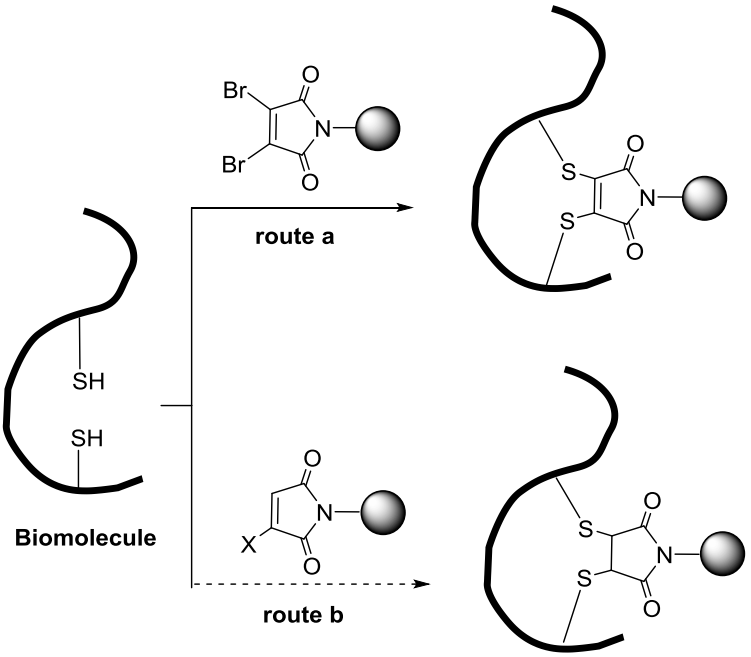
Scheme 81. Reaction between bromomaleimide **57**/chloromaleimide **86** and GSH

However, due to the signal to noise ratio, it was not possible to eliminate the possibility of side product formation, making the experiment inconclusive. The pH screen (pH 4 to 6) was performed in the attempt to slow down the reaction and also see if the intermediate succinimide can be detected but no signs of this intermediate were observed.

II.4 Succinimide bridging of disulphide bonds

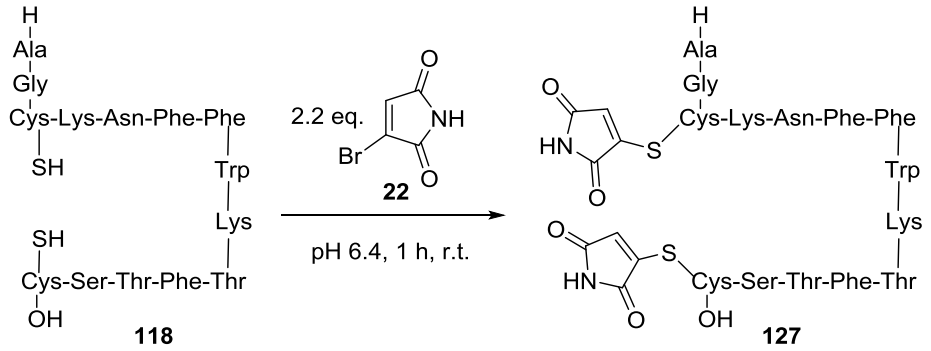
While the utility of disubstituted maleimides such as dibromomaleimide and dithiophenolmaleimide in disulphide labelling has been extensively explored, the use of monosubstituted maleimides has not been studied. The reaction between a reduced disulphide bond and a monosubstituted maleimide is expected to lead a dithiosuccinimide bridge as route b in Scheme 82, as opposed to disubstituted

maleimides such as dibromomaleimide and dithiomaleimide which lead to a maleimide bridge as in route a in Scheme 82. Such dithiosuccinimides are expected to have different properties to dithiomaleimides (e.g. different reactivity with thiols**,** hydrolytic stability etc.) and could serve as a complementary approach to disulphide bridging.



Scheme 82. Reactivity of disubstituted (route a) and monosubstituted (route b) maleimides towards a reduced disulphide

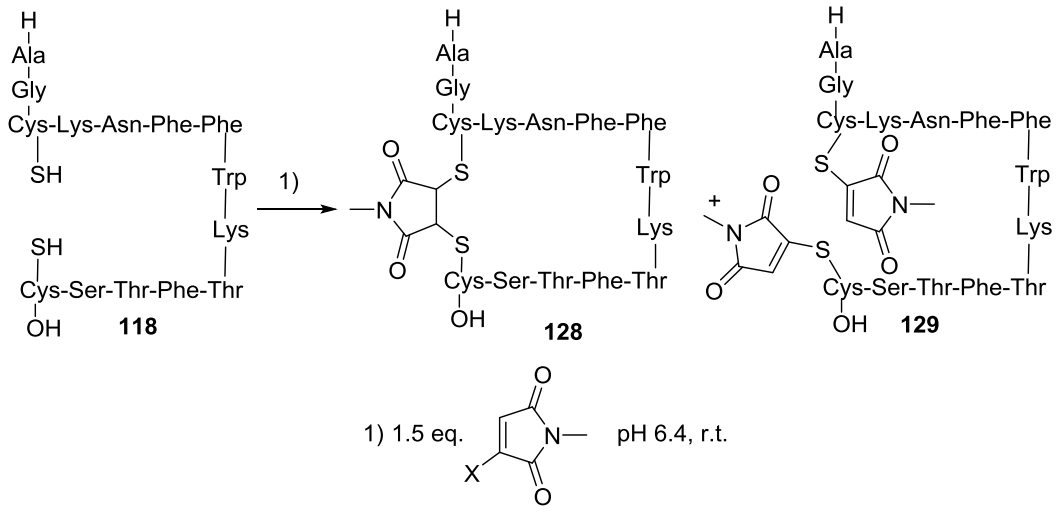
However, previous experiments conducted by L. Tedaldi, have shown that when 2.2 eq. of bromomaleimide **22** were reacted with reduced somatostatin **118**, the product of the reaction was exclusively the *bis*-labelled peptide **127** (M = 1834), (Scheme 83).[177] This suggests that the rate of addition of a second molecule of bromomaleimide **22** is higher than the rate of the intramolecular thiol attack on the thiomaleimide.



Scheme 83. Reaction between reduced somatostatin and bromomaleimide **22**

Having shown that phenoxymaleimides display a lower reactivity towards cysteine groups in peptides and proteins, we envisaged that this new class of reagents would also display a different reactivity pattern towards disulphide bonds compared to the highly reactive bromomaleimides.

To test this hypothesis, reduced somatostatin **118** was treated with various amounts of bromomaleimide **57** and phenoxymaleimide **101** respectively (Fig. 32).



57 (X = Br) **101** (X = OPh)

X	Eq.	Time (min)	Yield 128 (%)	Yield 129 (%)
Br	1.5	10	70	30
OPh	1.5	10	100	0
Br	2.5	60	22	78
OPh	2.5	60	100	0
OPh	10	60	100	0

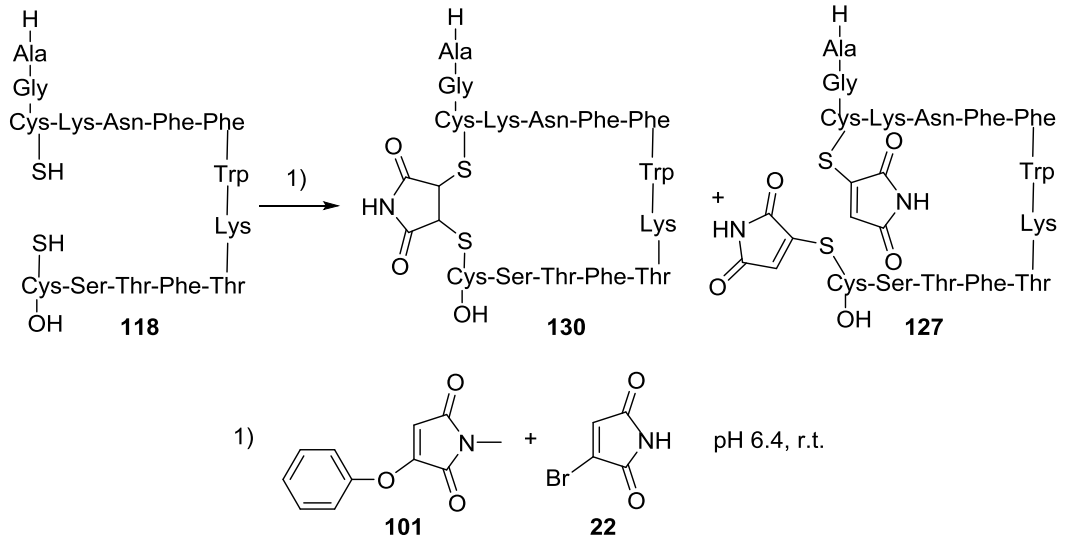
Fig. 32. LC-MS based analysis of reactions between monosubstituted maleimides **57** and **101** and reduced somatostatin

When 1.5 eq. of each of the reagents were used, the reaction with bromomaleimide **57** led to a mixture of succinimide bridged conjugate **128** (M = 1750) and *bis*-labelled adduct **129** (M = 1862) in a 7 : 3 molar ratio, while the reaction with phenoxymaleimide lead only to the succinimide bridged conjugate **128**. The reactions were repeated in the presence of a larger excess of reagent (2.5 eq.). In the case of the bromomaleimide reaction, the same product mixture was obtained

but this time the distribution of the product was inverted: 78% *bis*-labelled conjugate **129** and 22% succinimide bridged adduct **128**. Increasing the amount of phenoxymaleimide **101** to 2.5 eq. and 10 eq. led to no change in the reaction outcome, the only product detected being the succinimide bridged conjugate **128** (Fig. 32).

These results indicate that the bromomaleimide **57** prefers to add to the two cysteines rather than undergo the intramolecular cyclisation leading to the succinimide bridged adduct **128.** Phenoxymaleimide **101**, being less reactive, reacts with one of the two cysteine groups and then the double bond of the maleimide is attacked by the second cysteine in a conjugate addition type reaction. The experimental results show that the intermolecular second addition of a phenoxymaleimide molecule to the free cysteine leading to **129** is slower than the intramolecular cyclisation leading to the succinimide bridged product **128**.

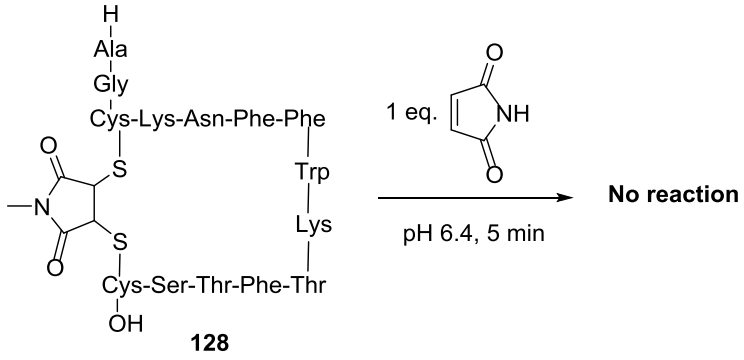
To support this first set of results, a competition experiment between bromomaleimide **22** and phenoxymaleimide **101** was designed (Fig. 33). First an equimolar mixture of **22** and **101** (1.5 eq of each, 1 : 1 molar ratio) was treated with reduced somatostatin **118** and then the reaction was repeated starting from a 1 : 5 molar mixture of **22** and **101** (1.5 eq. of **22** and 7.5 eq. of **101**). The different functionalisation of the nitrogen atom chosen allowed the identification of the maleimide that had reacted. In both cases, a mixture of the succinimide bridged adduct **130** (M = 1736) and the *bis*-labelled adduct **127** (M = 1834) was obtained and based on their masses, it was possible to conclude that the products are derived from the bromomaleimide addition only.



22 (eq.)	101 (eq.)	Time (h)	Yield 130 (%)	Yield 127 (%)
1.5	1.5	0.08	56	46
1.5	1.5	16	20	80
1.5	7.5	0.08	51	49
1.5	7.5	16	17	83

Fig. 33. Competition experiments between bromomaleimide **22** and phenoxymaleimide **101**

The easiest way to test for the presence of free thiols, and therefore confirm the completion of the succinimide bridging is to add a reactive electrophile, known to react with thiols, such as maleimide. The succinimide bridged somatostatin conjugate **128** was treated with 1 eq. maleimide. LC-MS after 5 min of incubation at room temperature showed no sign of any reaction, indicating the absence of free thiols in the system (Scheme 84).



128

Scheme 84. Reaction of succinimide bridged somatostatin conjugate **128** with maleimide

In conclusion, by using reagents of tuned reactivity, succinimide bridging of the reduced disulphide bond in somatostatin was achieved. At this point it is important to highlight the fact that as opposed to maleimide bridging, which affords a single product **131**, succinimide bridging affords **132** as a diastereoisomeric mixture of products (Fig. 34). In order to best make use of this strategy in biological systems, a very clear understanding of its structural properties will be required. From the small molecule work conducted so far, we anticipate that the *trans* isomers will be favoured but further investigation has not yet been conducted.

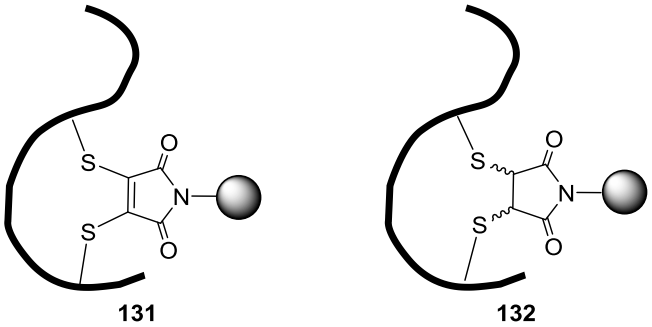


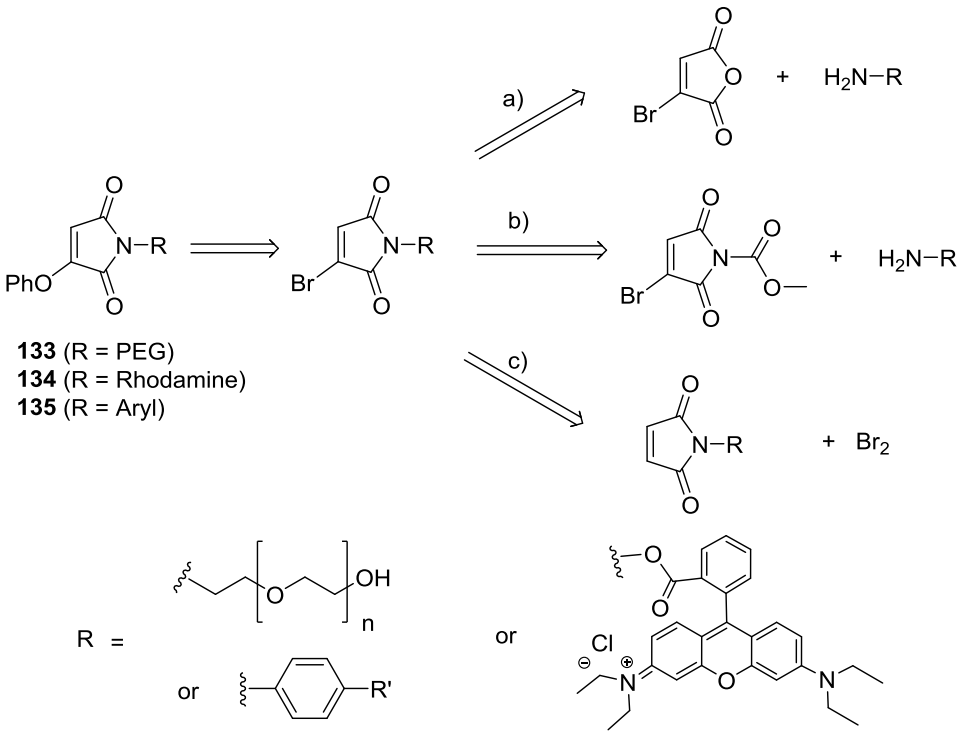
Fig. 34. Maleimide bridged biomolecule **131** and succinimide bridged biomolecule **132**

Having demonstrated that *N*-methyl phenoxymaleimide displays a high selectivity for succinimide bridging over the double addition, the next step was to test the scope of this bioconjugation approach and to prove its utility. For the latter aim, we set out to synthesise some useful *N*-functionalised phenoxymaleimides. The synthetic strategies employed are detailed in the next section.

II.4.1 Synthesis of *N*-functionalised phenoxymaleimides

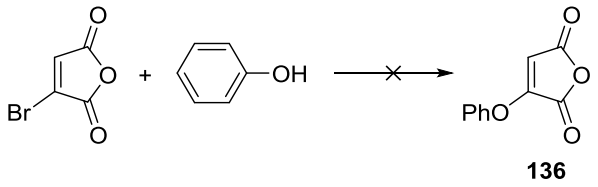
Commonly employed protein tags are PEG chains, used mainly to enhance *in vivo* stability and water solubility of compounds, and fluorophores, for visualisation purposes. We therefore decided to functionalise the nitrogen atom of phenoxymaleimide with a fluorophore and a mini-PEG chain respectively. Rhodamine B was the fluorophore of choice as it has very good properties for imaging: broad fluorescence in the red spectrum of visible light, high quantum yield, and good photostability.[195] Aromatic substituents on the nitrogen atom are known to induce changes in the electron distribution across the maleimide ring and therefore increase the reagent's susceptibility to hydrolysis; consequently a representative of *N*-aryl phenoxymaleimides was targeted.

The targeted compounds were **133** - **135** (Scheme 85); the original synthetic plan involved firstly the nitrogen functionalisation of bromomaleimide and a subsequent conjugate addition reaction with phenol. The bromomaleimide would be obtained *via* either a condensation reaction between the bromomaleic anhydride and the appropriate amine (Scheme 85, route a), starting from the activated *N*-methoxycarbonyl bromomaleimide, by addition of the amine (Scheme 85, route b) or from the already functionalised maleimide, by bromination and subsequent dehydrobromination (Scheme 85, route c).



Scheme 85. Retrosynthetic routes for the synthesis of *N*-functionalised phenoxymaleimides

A one step procedure starting from the phenoxymaleic anhydride **136** is not possible as it is anticipated that its synthesis would be highly disfavoured. This is due to the expected high selectivity of phenol (hard nucleophile) for the attack on the carbonyl groups (hard electrophile) of the bromomaleic anhydride rather than on the double bond (soft electrophile), (Scheme 86).

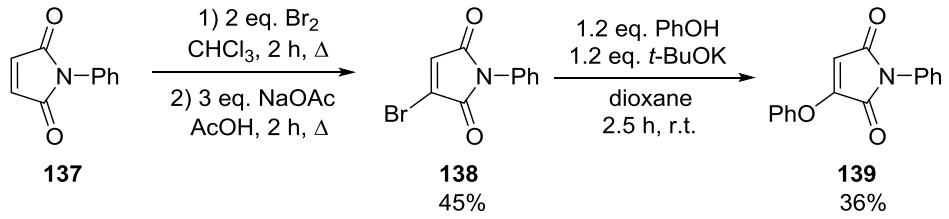


136

Scheme 86. Reaction between phenol and bromomaleic anhydride

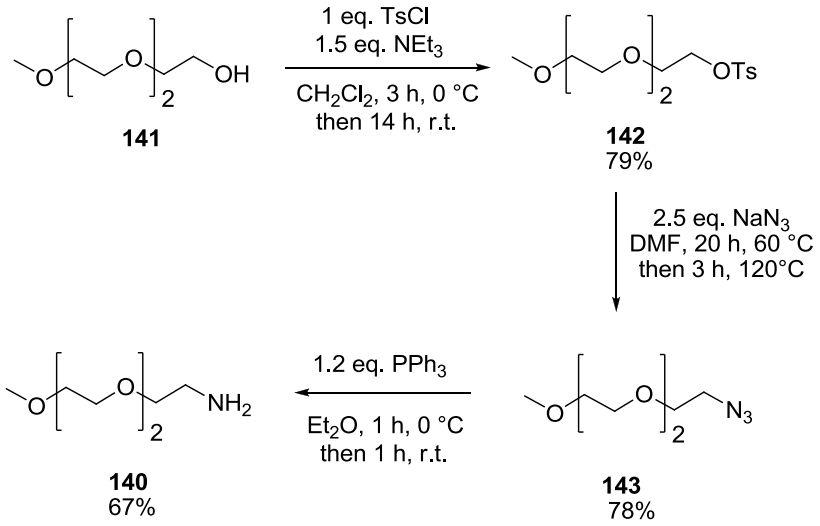
Following the route described in Scheme 85c, *N*-phenyl phenoxymaleimide **135** was synthesised in two steps starting from commercially available *N*-phenyl maleimide **137**. The bromination of **137** was performed under standard conditions, while the dehydrobromination was achieved using the acid-catalysed mechanism described in Chapter II.1. The conjugate addition of phenol to the *N*-phenyl bromomaleimide **138** was successful but afforded the desired product **139** in a modest yield (36%), (Scheme 87). The low yield is associated with repeated column

chromatography to obtain pure product and also to the formation of side products, as indicated by the TLC of the reaction mixture.



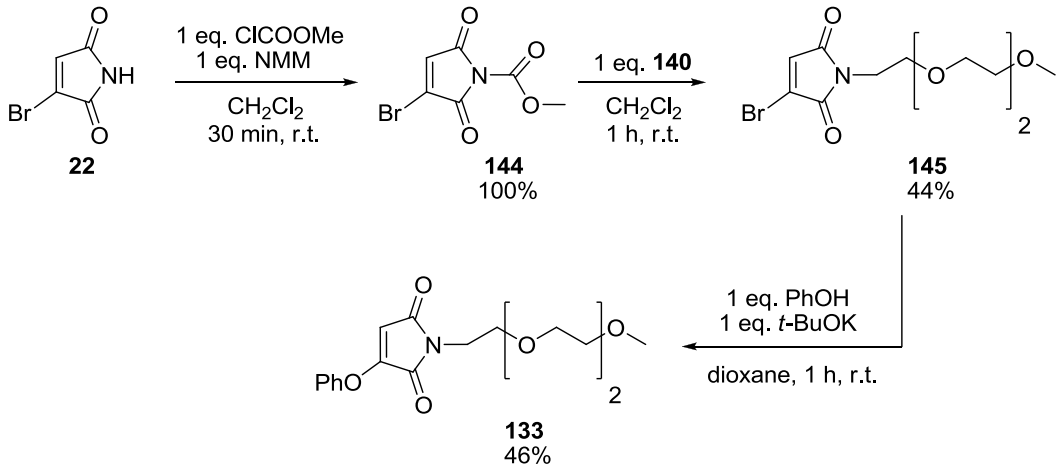
Scheme 87. Synthesis of *N*-phenyl phenoxymaleimide **139**

For the preparation of an *N*-PEGylated analogue it was decided to use a 3 unit PEG chain, which has the advantage of being homogenous and therefore enables ^{1}H-NMR analysis of the product. The 3 unit PEG amine **140** was prepared in three steps with an overall yield of 41%, according to a literature procedure (Scheme 88).[196] The commercially available PEG chain **141** was reacted with tosyl chloride, to give the activated tosylated analogue **142**. The S_N2 displacement with sodium azide required heating for an extended time period and excess reagent due to the weak nucleophilicity of the sodium azide. In the end, the desired PEG-amine **140** was obtained *via* a Staudinger reduction, using triphenylphosphine.



Scheme 88. Synthesis of PEG amine **140**

With the PEG-amine **140** in hand, the synthesis of the *N*-PEG phenoxymaleimide **133** was straightforward, starting from the activated *N*-methoxycarbonyl bromomaleimide **144** (Scheme 89).

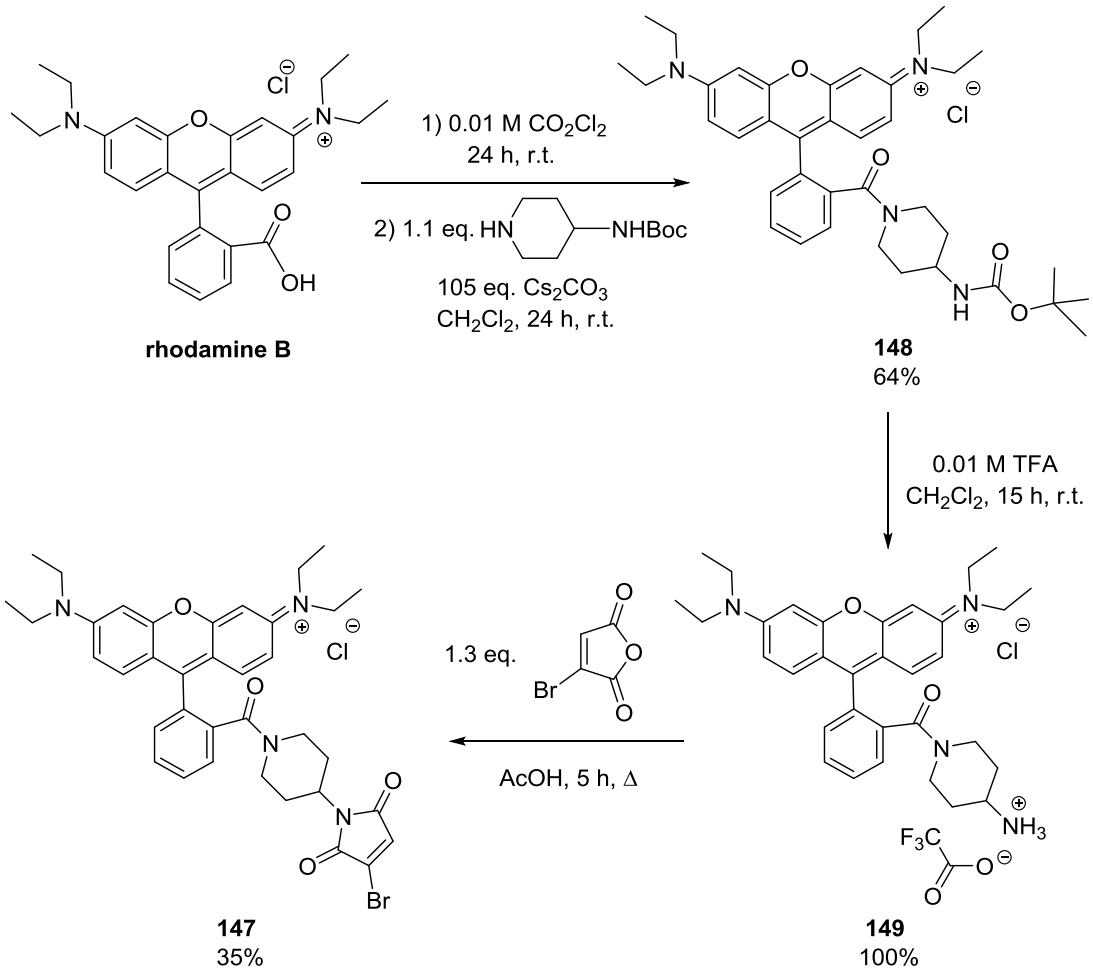


22 → **144** 100% → **145** 44% → **133** 46%

Scheme 89. Synthesis of *N*-PEG phenoxymaleimide **133**

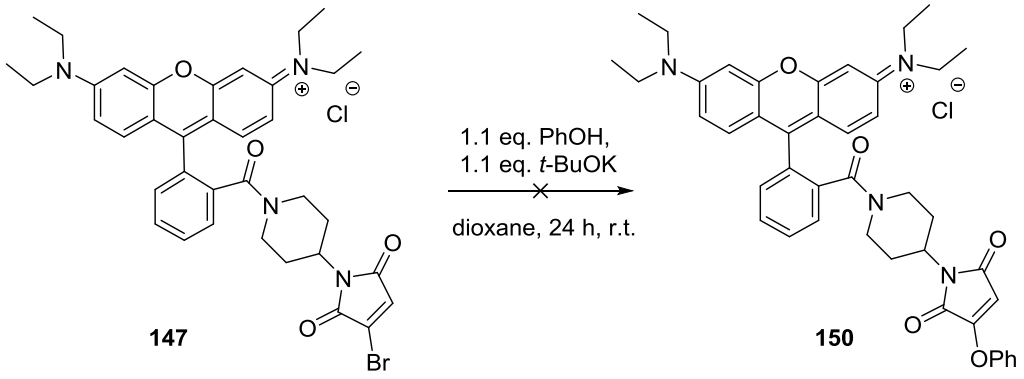
This synthetic route was developed in the Baker group and represents a mild method for the synthesis of *N*-functionalised bromomaleimides, avoiding the harsh conditions required by the two other routes (routes a and c in Scheme 85).[197] This route is particularly useful when the amine contains sensitive functionalities. Another advantage of this route is the ease of synthesising the *N*-methoxycarbonyl bromomaleimide **144**; this is obtained in quantitative yield from bromomaleimide **22** by addition of methyl chloroformate in the presence of *N*-methyl morpholine. The product is not stable during flash chromatography on silica gel but this kind of purification is not required, as after the extraction with CH_2Cl_2 the product is free of impurities. The nucleophilic displacement with the amine to give *N*-PEG bromomaleimide **145** proceeded with a yield of 44% and lastly, the bromide was displaced by phenol, under the standard conditions detailed in Chapter II.1.

The final targeted phenoxymaleimide was the *N*-rhodamine labelled derivative **134**. Having a literature precedent for the synthesis of the *N*-rhodamine labelled bromomaleimide **147**,[149] it was decided to prepare **147** and then attempt the conjugate addition with phenol (Scheme 90). Rhodamine-bromomaleimide derivative **147** was generated by condensation of the bromomaleic anhydride with intermediate **149**. The low yield in the final step is associated with difficulties in purification.



Scheme 90. Synthesis of *N*-rhodamine bromomaleimide **147**

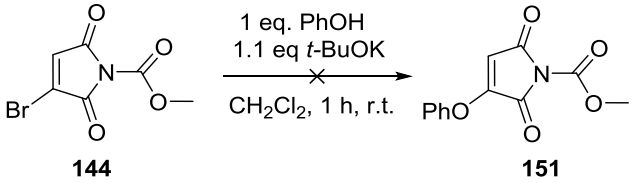
Unfortunately, the conjugate addition with phenol on bromomaleimide **147** was unsuccessful and the starting material was fully recovered (Scheme 91).



Scheme 91. Conjugate addition of phenol to bromomaleimide **147**

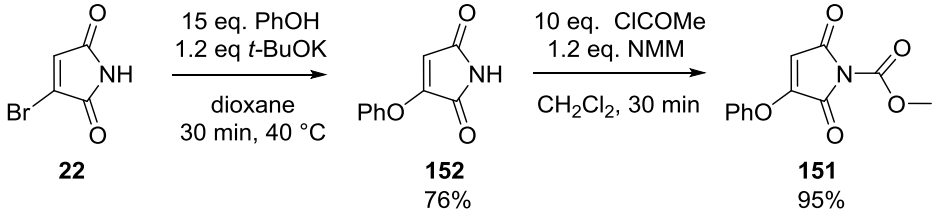
The next strategy that was explored was the route involving the carbamate chemistry, which required *N*-methoxycarbonyl phenoxymaleimide **151** to be synthesised. Unfortunately, the addition of phenol to *N*-methoxycarbonyl

bromomaleimide **144** did not lead to the desired product **151** (Scheme 92). This is likely due to the competing addition of the phenoxide, a hard nucleophile, to the carbonyls.



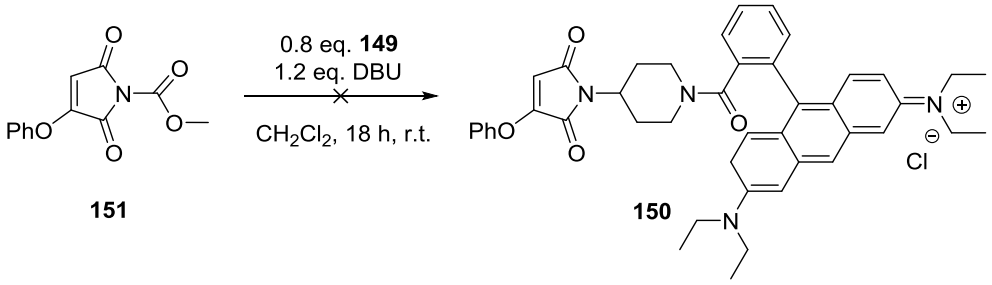
Scheme 92. Conjugate addition of phenol to *N*-methoxycarbonyl bromomaleimide **144**

This led to the necessity of synthesising phenoxymaleimide **152** followed by its activatation with methyl chloroformate (Scheme 93). Initial attempts to displace the bromide in **22** with phenol led to a complex mixture of products, likely caused by the oligomerisation initiated by deprotonation of the imide. The problem was solved by performing the reaction at a high concentration of phenol (17 mM) and increasing the temperature to 40 °C. Under these conditions, the carbamate could then be installed in an excellent yield (95%).



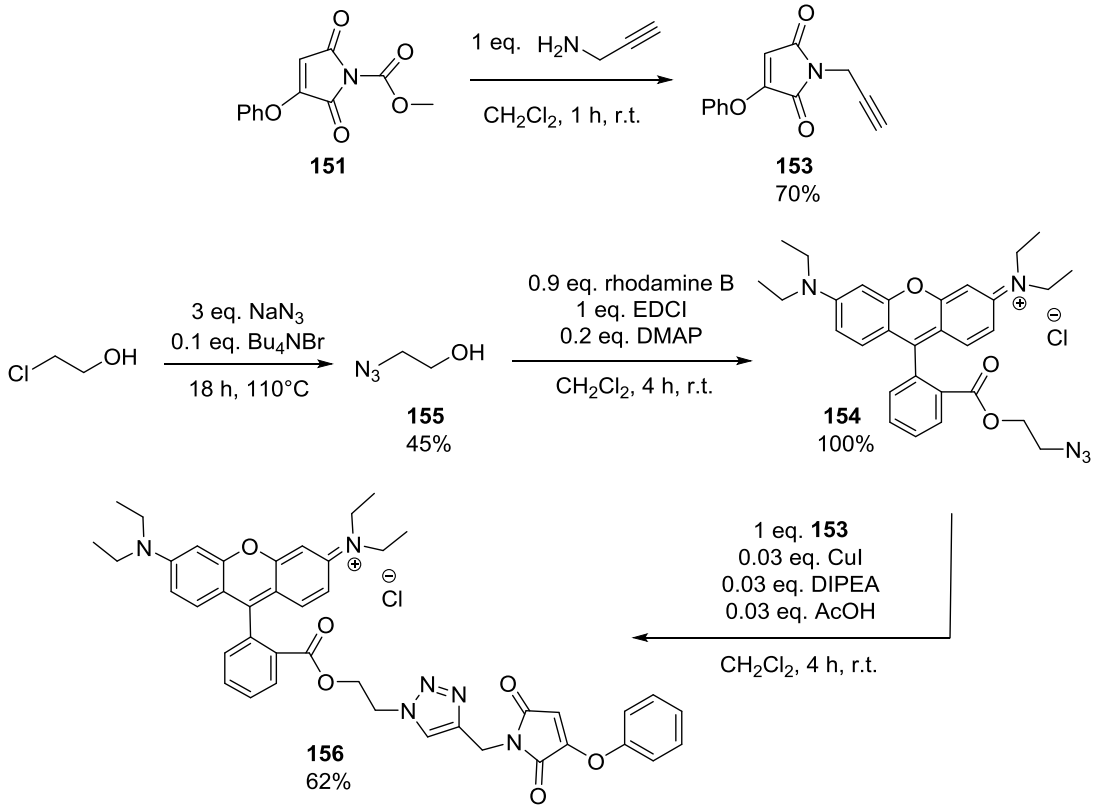
Scheme 93. Synthesis of *N*-methoxycarbonyl phenoxymaleimide **151**

Unfortunately, the reaction between **151** and the rhodamine-amine derivative **149** was unsuccessful (Scheme 94). The reaction was performed in the presence of DBU as a base, which was required to free the amine in **149**. This result was however not very surprising as previous results within the Baker group[198] indicated the *N*-methoxycarbonyl substituted maleimides tend to react less readily with bulky amines.



Scheme 94. Reaction between phenoxymaleimide **151** and rhodamine amine **149**

It was then decided to attempt the labelling of phenoxymaleimide with rhodamine using the Huisgens [3+2] cycloaddition reaction. The alkyne functionality required for the click reaction was easily installed on the nitrogen atom of the phenoxymaleimide, by reacting the *N*-methoxycarbonyl phenoxymaleimide **151** with propargyl amine (Scheme 95). A literature search provided a highly efficient route towards azido-rhodamine derivative **154**,[199] starting from rhodamine B and azidoethanol **155**,[200] which in turn is synthesised by nucleophilic displacement with sodium azide from commercially available chloroethanol (Scheme 96). With both the alkyne and azide components in hand, the click reaction was attempted using an adapted literature protocol,[201] and gratifyingly yielded the desired *N*-rhodamine labelled phenoxymaleimide **156** in a good yield (62%).



Scheme 95. Convergent synthesis of *N*-rhodamine phenoxymaleimide **156**

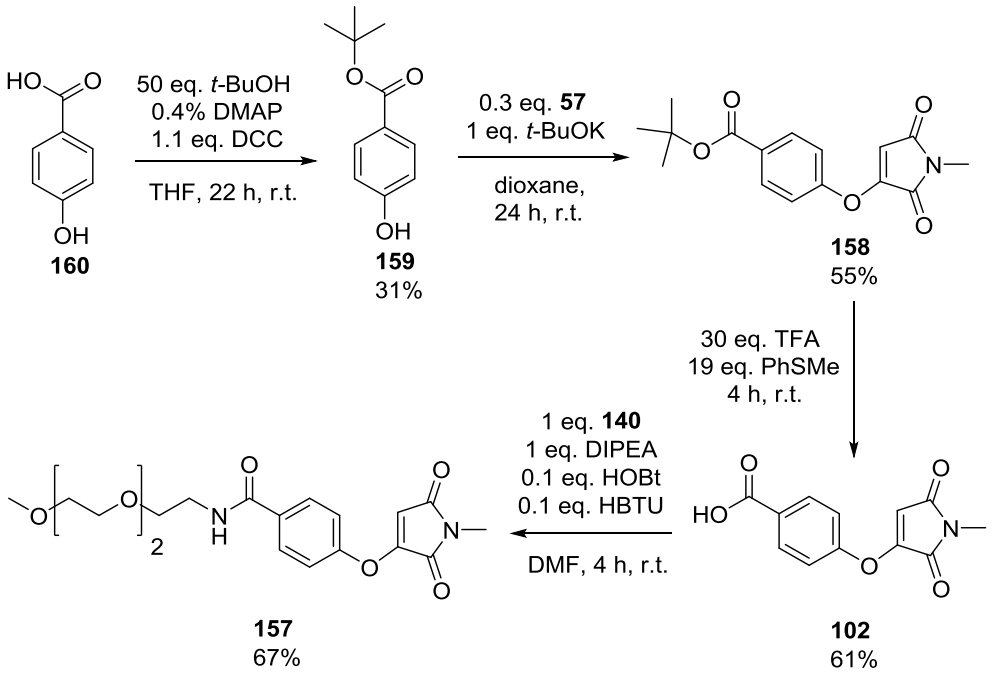
II.4.2 Synthesis of water soluble phenoxymaleimides

Water solubility is a key feature of protein labelling reagents as it contributes to the preservation of the stability of the biomolecule by allowing the transformation to take place in buffer. The aim was to synthesise a water soluble phenoxymaleimide derivative. The typical way of increasing the water solubility of an

organic compound is by decorating it with hydrophilic groups such as carboxylic acids (COOH), PEG chains ($(OCH_2CH_2)_n$) or sulfonates ($(SO_3)^{2-}$).

The following work is the result of a collaboration with Masters's student Patrick Mayer. It was decided to install the hydrophilic tag on the phenyl ring in order to retain the three points of attachment of phenoxymaleimide for further conjugation. We first set out to test the influence on the water solubility of a carboxylic group and a mini-PEG chain respectively.

Following the synthetic route developed by Masters's student P. Mayer, the water soluble phenoxymaleimide **157** was prepared in a 6.9% overall yield, 2.9% higher than the one previously reported.[202] The synthesis involves the synthesis of the protected carboxylic acid phenoxymaleimide **158** under standard conditions *via* conjugate addition of phenol **159** to bromomaleimide (55%). Phenol **159** was prepared according to a literature procedure (Scheme 96).[203] Compound **158** was then subjected to standard deprotection conditions (32 eq. TFA, 19 eq. thioanisole, 4 h, CH_2Cl_2)[204] to give the desired *p*-carboxylic acid phenoxymaleimide **102** in a satisfactory yield (61%). The synthesis was completed by a HBTU/HOBt amide coupling with the already prepared PEG-amine **140**.



Scheme 96. Synthesis of water-soluble phenoxymaleimides **157**

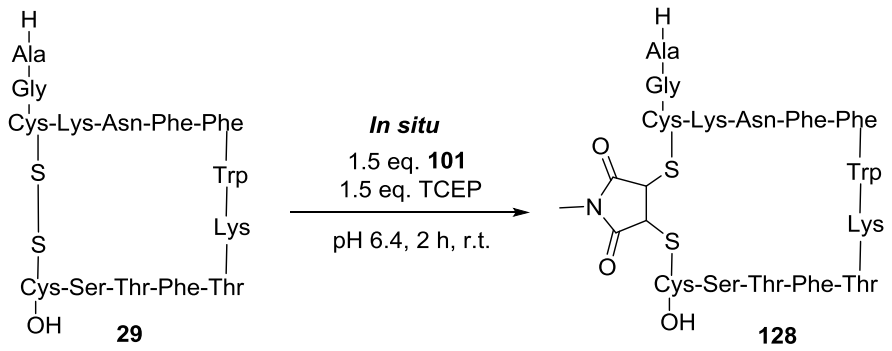
The water solubility of the tested reagents was measured by Patrick Mayer in pH 6.4 buffer (50 mM sodium phosphate), as this was the pH used for the disulphide

bridging of somatostatin. Phenoxymaleimide **157** displayed a solubility of 1.20 mmol/mL which was compatible with the succinimide bridging protocol used so far.

II.4.3 *In situ* succininimide bridging of disulphide bonds

In analogy to the maleimide bridging of disulphide bonds developed within our group, we were interested to see whether the succinimide bridging could be performed *in situ*. This means that the reducing agent is added to a mixture of the peptide and the labelling reagent so that as soon as the disulphide bond is reduced it reacts with the labelling reagent. In this way, the time the disulphide bond stays in its reduced form is minimised.

To test this, a sample of somatostatin **29** (200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was mixed with phenoxymaleimide **101** (1.5 eq.) and TCEP (1.5 eq.) was added to the resulting mixture (Scheme 97). LC-MS analysis after 2 h at room temperature indicated full conversion of native somatostatin **29** to the succininimide bridged product **128**.



Scheme 97. *In situ* succinimide bridging of somatostatin

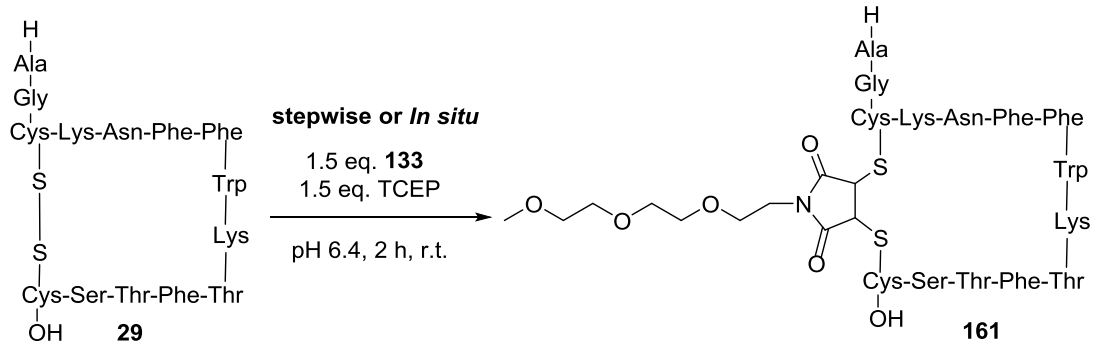
Phenoxymaleimides are the first of the reagents tested so far within the library to display no cross-reactivity with TCEP. Therefore they are ideal reagents for the *in situ* protocol.

II.4.4 Scope of the stepwise succinimide bridging of disulphide bonds

Having demonstrated that the reaction between reduced somatostatin and phenoxymaleimide (1.5 - 10 eq.) leads rapidly and quantitatively to the succinimide bridged peptide, we wanted to explore the scope of this strategy regarding to the protein substrate and also prove its utility in the area of protein modification by using reagents bearing useful tags (*e.g.* fluorophores, PEG chains).

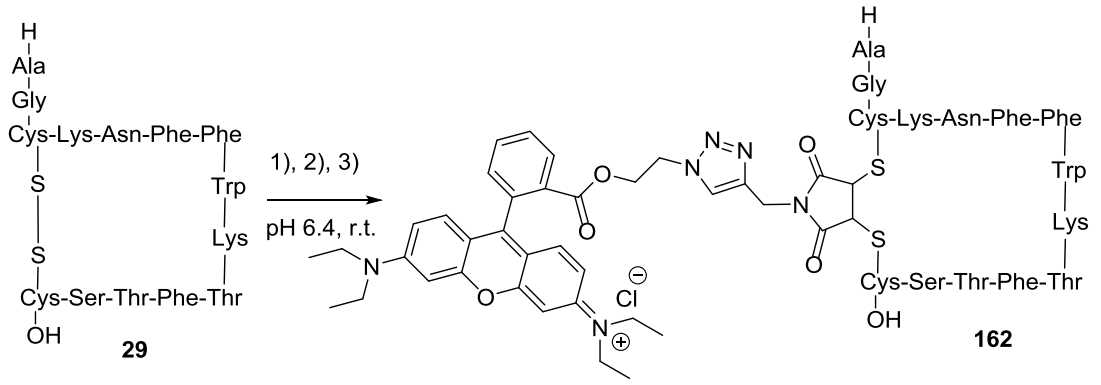
Firstly, the successful bridging of somatostatin with *N*-functionalised phenoxymaleimides was shown.

The two reagents investigated were the *N*-pegylayed and rhodamine labelled phenoxymaleimides **133** and **156**. The pegylation proceeded smoothly both stepwise and *in situ*, with equimolar amounts of the reducing and labelling reagents (1.5 eq. each), (Scheme 98).



Scheme 98. PEGylation of somatostatin

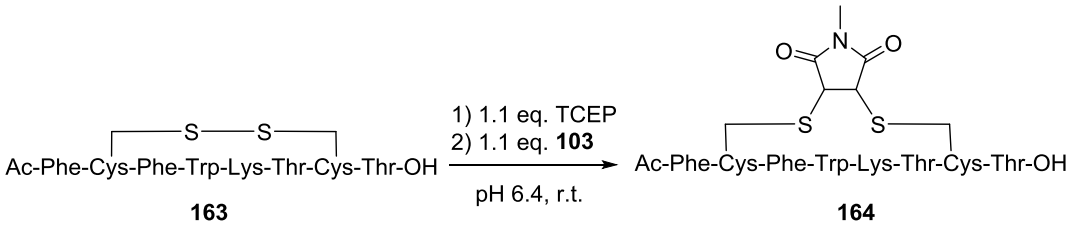
Next, labelling with the *N*-rhodamine labelled phenoxymaleimide **156** was achieved. The transformation required developing an optimized protocol consisting of the addition of phenoxymaleimide **156** (1.2 eq.) to reduced somatostatin followed by addition of an additional portion of TCEP (0.6 eq.) after 30 min of incubation at room temperature (Scheme 99).



1) 1.2 eq. TCEP, 1 h; 2) 1.2 eq. **156,** 30 min; 3) 0.6 eq. TCEP, 30 min

Scheme 99. Stepwise succinimide bridging of somatostatin with *N*-rhodamine phenoxymaleimide **156**

Another peptide studied within the group is the peptide octreotide, which is the clinical analogue of somatostatin, marketed by Roche and mainly used for the treatment of acromegaly.[205] Octreotide **163** is a 8-amino acid peptide and is supplied as the acetate salt. Masters student Hanno Kossen showed that indeed, the succininimide bridging of octreotide can be achieved under very similar conditions to the ones described for somatostatin (Scheme 100).[206]



163 **164**

Scheme 100. Succinimide bridging of octreotide with phenoxymalemide **103**

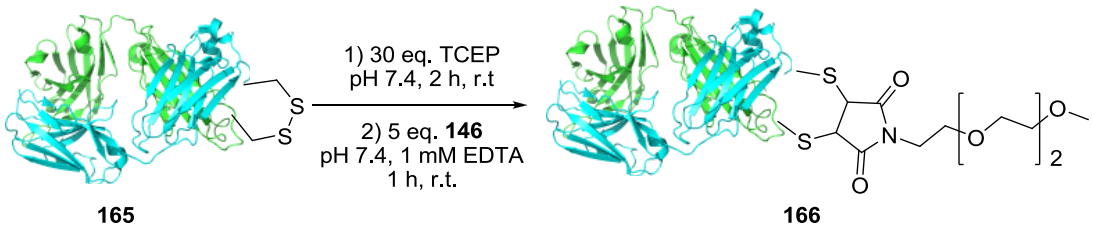
The next step was to investigate the applicability of this method on large proteins. Antibody fragments are now highly valued for their utility in therapeutics[207,208] (*e.g.* synthesis of antibody-drug conjugates, tumor imaging) and therefore their labelling with useful functionalities (e.g. drugs, fluorophores, PEG chains) would bebeneficial. The model systems chosen were Trastuzumab Fab, Rituximab Fab and the shMFE disulphide-stabilised variable fragment (ds-scFv), each containing a single disulphide bond.

Rituximab (Rituxan ®, Genentech) is a chimeric monoclonal antibody that targets the human CD20 cell surface antigen.[209] It has received FDA approval to be used as a therapeutic reagent for non-Hodgkin's lymphoma and rheumatoid arthritis. Trastuzumab (Herceptin) is a monoclonal IgG1 antibody that binds to HER2/neu receptor and has received FDA approval for the treatment of certain breast and gastric cancers.[210] The disulphide bond of interest in Fab fragments is between the heavy and light chains and is distal to the antigen-binding site. The ds-scFv fragment used has been developed by the group of Prof. Chester (UCL Cancer Institute) and targets the carcinoembrionic antigen (CEA). The two domains are linked by a artificially inserted peptide linker and a disulphide bond, for extra stability.[211] This particular interdomain disulphide bond is our target for the succinimide bridging.

Rituximab Fab was provided by Dr. Felix Schumacher[187] in the Baker group at UCL and the anti-CEA ds-scFv by Dr. Berend Tolner in the Chester group at UCL Cancer Institute. Trastuzumab Fab was prepared by pepsin and papain digest, following a literature protocol.[212]

Rituximab Fab **165** was successfully pegylated with phenoxymaleimide **146** (Scheme 101). The two step procedure, also referred to as the "stepwise protocol", begins with the reduction of the protein using TCEP (30 eq.). After completion of the reduction step (which can be checked by LC-MS), the protein was buffer swapped into PBS (pH 7.4) containing EDTA (1 mM), to remove the unreacted reducing agent and avoid reoxidation of the disulphide bond. The labelling reagent **146** (5 eq.) was

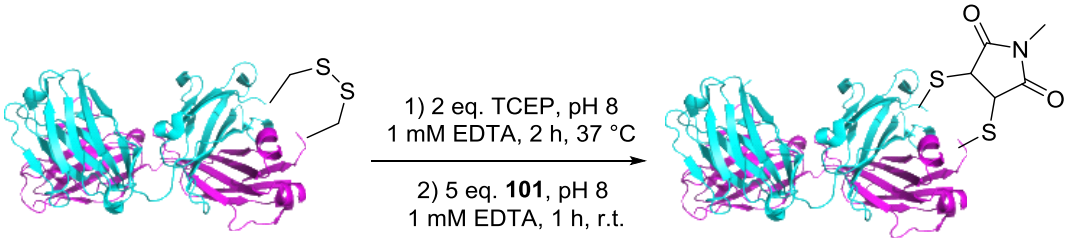
then added and the resulting mixture was incubated for 1 h at room temperature. This afforded the succinimide bridged Rituximab Fab **166** as the sole product of the reaction.



165 **166**

Scheme 101. Stepwise succinimide bridging of Rituximab Fab with phenoxymaleimide **146**

Similarly, Herceptin (Trastuzumab) Fab **167** was successfully labelled using phenoxymaleimide **101** (Scheme 102). The main difference in the protocol compared with that for Rituximab Fab **165** is in the reduction step, which was performed in this case in pH 8 borate buffer and at 37 °C with just 2 eq. of TCEP. No purification between steps was necessary hence the labelling reagent **101** (2 eq.) was added immediately after the reduction had been confirmed by LC-MS. This protocol is referred to as “sequential bridging”.

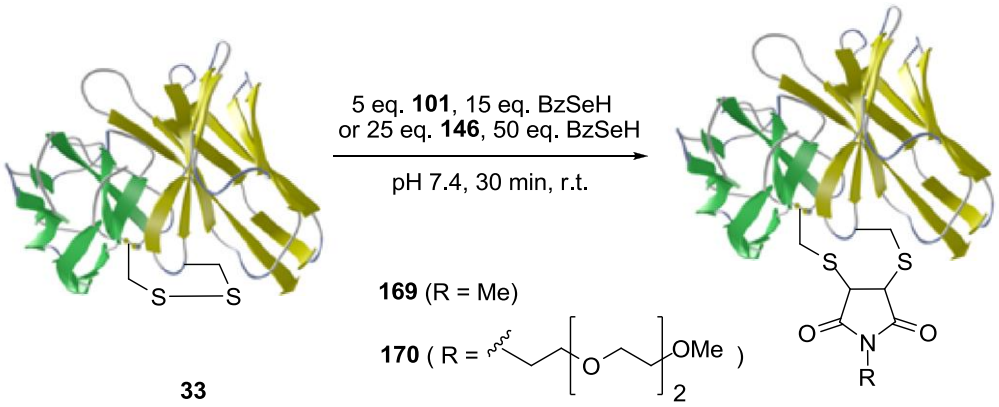


167 **168**

Scheme 102. Sequential succinimide bridging of Trastuzumab Fab with phenoxymaleimide **101**

Finally, the disulphide bond stabilised scFv (ds-scFv) **33** was labelled with **101** and **146.** The bridging protocol was adapted from the one reported by Dr. Schumacher in the Baker group.[187] The bridging was performed *in situ* and consists of the addition of the labelling regent (5 eq. in the case of **101** and 25 eq. in the case of **146**) and of reducing agent, benzeneselenol (15 eq. for the reaction involving **101** and 50 eq. for the reaction involving **146**) simultaneously (Scheme 103). The increased amounts of labelling and reducing reagents required when phenoxymaleimide **146** was used can be correlated to the larger size of this reagent compared to the *N*-methylated analogue **101** and to the fact that the disulphide bond is not very solvent-exposed. This is in accordance with the observations of Dr.

Schumacher in the Baker group who observed the lack of reactivity of the ds-scFv towards TCEP as a result of the steric bulk of TCEP.

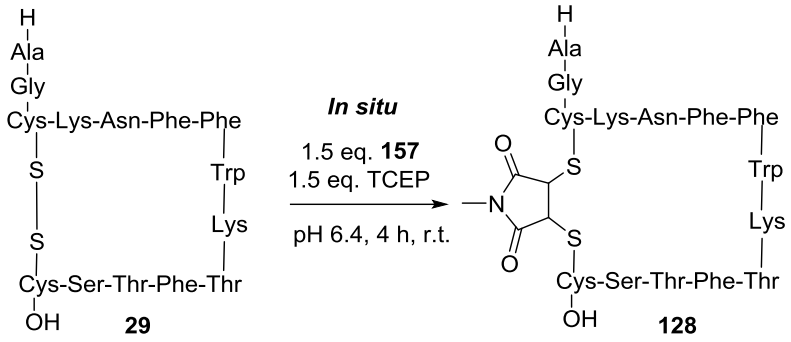


Scheme 103. Succinimide bridging of ds-scFv

To highlight the utility of phenoxymaleimides, the reaction between ds-scFv **33** and bromomaleimide **57** was performed under the same conditions. This reaction afforded just unreacted starting material, due to the cross-reactivity between bromomaleimide and benzeneselenol.

II.4.5 Organic solvent free succinimide labelling of somatostatin

As mentioned before, water soluble reagents for protein labelling are highly sought after due to the concern that the addition of organic solvents to proteins can disturb their tertiary structures and therefore interfere with their biological activity. Phenoxymaleimide **157** was designed as a water soluble representative of this class of compounds, and it was shown to have a solubility of 10 mM in pH 6.4 50 mM phosphate buffer, at room temperature. It was chosen to be the ideal candidate for attempting the succinimide bridging of somatostatin **29** under organic solvent free conditions. Consequently, a solution of somatostatin (200 μM, pH 6.4 50 mM phosphate buffer) was mixed with phenoxymaleimide **157** (1.5 eq., 10 mM stock solution in the same buffer) and the water soluble reducing agent TCEP added (Scheme 104). LC-MS analysis of the reaction mixture indicated completion of the reaction after 4 h of incubation at room temperature.



Scheme 104. *In situ* organic solvent-free succinimide bridging of somatostatin

This is the first example of a completely organic free disulphide bridging reaction involving substituted maleimides.

II.4.6 Stability of the succinimide bridged conjugates towards thiols

The stability of protein conjugates towards thiols is of significant importance especially in the case of protein drug conjugates which must survive plasma conditions in order to reach the targeted site. Body plasma contains several species with free thiol groups such as free cysteine, homocysteine, reduced glutathione and albumin,[213] while the intracellular environment is particularly rich in glutathione, which can reach concentrations of up to 11 mM.[214]

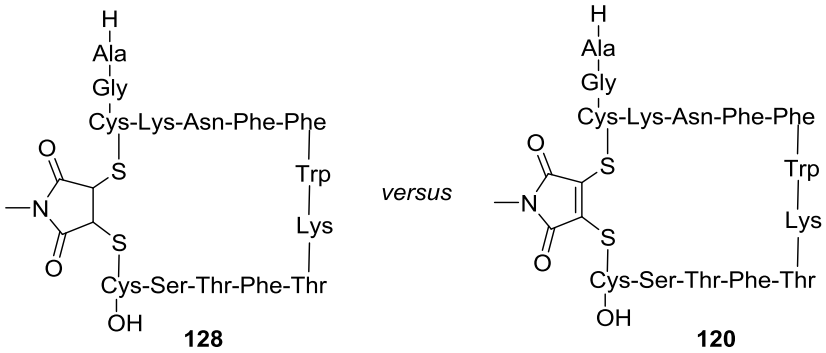
The stability of succinimide bridged conjugates towards thiols was assessed in parallel to the corresponding maleimide bridged constructs. As mentioned in the introduction chapter, the maleimide bridge undergoes cleavage in the presence of excess thiols such as 2-mercaptoethanol or glutathione.[139] However, when hydrolysed, the resulting maleamic acid conjugates are no longer susceptible to thiol attack.[141]

For the following experiments, somatostatin **29** and ds-scFv **33** were used as model systems.

First, somatostatin conjugates **128** and **120** were prepared as 200 µM solutions in pH 6.4 phosphate buffer containing 40% CH_3CN and 2.5% DMF, according to the general procedures for the stepwise bridging of somatostatin with phenoxymaleimides and dibromomaleimide respectively (Experimental section). Then a large excess (100 eq.) of 2-mercaptoethanol was added and the resulting solutions kept on ice for 13 h. Fig. 35 shows the percentage of cleavage to the reduced peptide undergone by the two constructs over time. The maleimide bridged conjugate **120**, expected to undergo rapid cleavage in the presence of such a large excess of thiol, did indeed lose the maleimide tag (80% degradation) within the first

hour. In the same time, the succinimide bridged somatostatin conjugate **128** was stable. Even after 13 h, LC-MS analysis of the sample containing succinimide bridged somatostatin conjugate **128** indicated that just 13% of **128** underwent cleavage. The small difference between the 1 h and the 13 h time points for conjugate **128** can be explained by the oxidation of the 2-mercaptoethanol, over time, to the corresponding disulphide.

The same experiment was carried out using reduced glutathione. This is of particular significance, considering the abundance of glutathione in the body. Fig. 35 shows the extent of cleavage of conjugates **120** and **128** when placed in a medium with a high concentration of thiol. This is consistent with the previous set of results. In this case however, the succinimide bridged somatostatin **128** does not undergo any degradation, even after 13 h on ice. This can be explained by the fact that glutathione, being more bulky, is slightly less reactive than 2-mercaptoethanol.



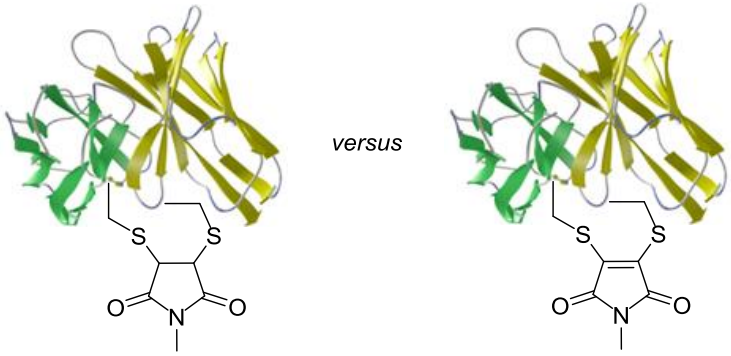
128 **120**

Thiol	Time (h)	Cleaved to reduced somatostatin 118 (%)	
		128	**120**
2-Mercaptoethanol	1	0	80
	13	13	85
Glutathione	1	0	16
	13	0	80

Fig. 35. LC-MS based thiol stability analysis of somatostatin conjugates **128** and **120**

The thiol stability of the maleimide bridged ds-scFv **171** has been studied and reported by Schumacher *et al.;*[146] a direct comparison with the corresponding succinimide analogue was desirable. For this, the succinimide bridged construct **169** was synthesised. In his study, Dr. Schumacher reported complete cleavage of the maleimide bridge after 48 h at ambient temperature in the presence of a large

excess (100 eq.) of glutathione or 2-mercaptoethanol (Fig. 36). On the other hand, the succinimide bridged construct **169** was completely stable even after the 48 h incubation.



169 **171**

Thiol (100 eq.)	Time (h)	Cleaved to reduced somatostatin 118 (%)	
		169	**171**
2-mercaptoethanol	48	0	100
Glutathione		0	100

Fig. 36. LC-MS based thiol stability analysis of ds-scFv conjugates **169** and **171**

In conclusion, for substrates containing a less accessible disulphide bond, such as ds-scFv **33** explored in this work, the succinimide tag is likely to have a longer *in vivo* half-life compared to the maleimide tag. The two approaches have complimentary applications; while the quick cleavage under high thiol concentrations of the maleimide tag can be used for drug-release into cells, the succinimide version will provide a slower or negligible release, depending on the time frame considered. The succinimide bridging can potentially be used for applications where irreversibility of labelling is desired, such as fluorescent tagging for visualisation purposes.

Encouraged by these results, the next experiment was designed to assess the stability of the two somatostatin conjugates **128** and **120** under cytoplasm mimicking conditions. Conjugates **128** and **120** were prepared as before and then dialysed into 20 mM HEPES buffer (100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4). The concentration of the peptide conjugates was adjusted to 100 μM and the resulting reaction mixtures were incubated at 37 °C for 21 h in the presence of 1 mM reduced glutathione. As we can see from Fig. 37, the succinimide bridged construct

128 is indeed more stable than the maleimide bridged analogue **120**. After 21 h, there was no more **129** and around 27% **128** (results not shown on the graph). However, this set of results shows that at 37 °C, the relevant temperature for *in vivo* applications, even the succinimide bridge undergoes cleavage of the tag.

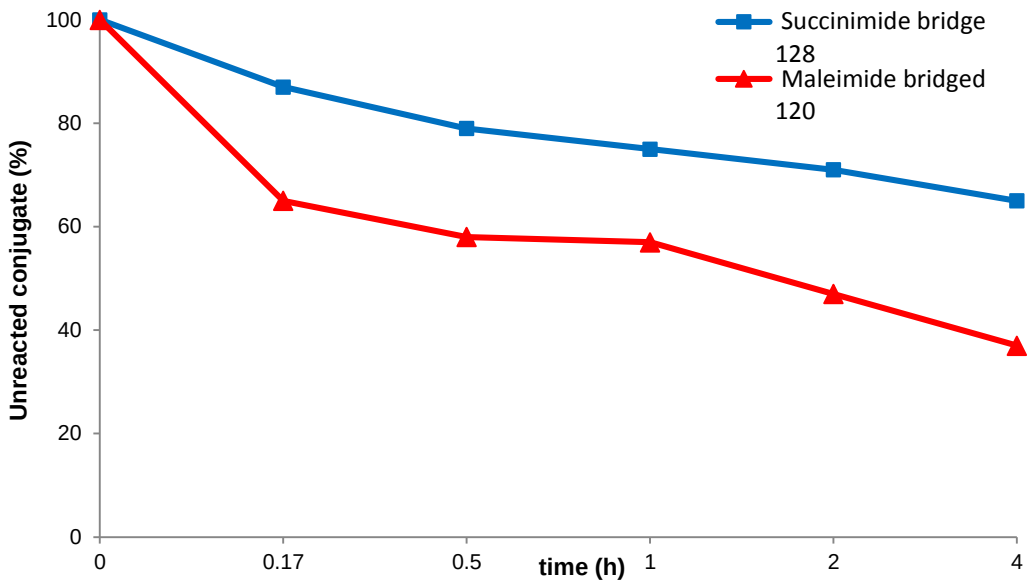
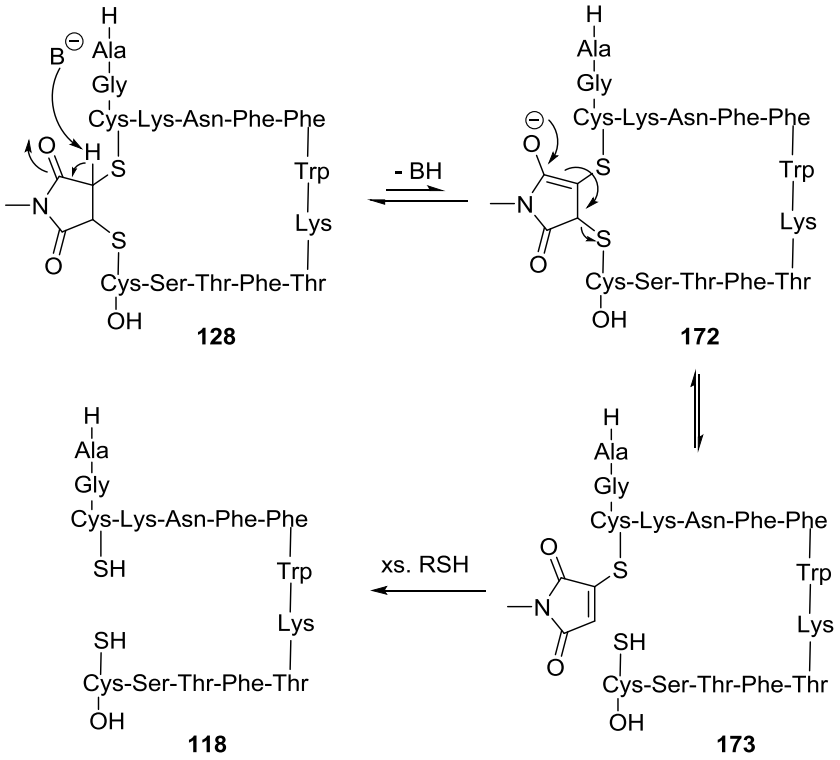


Fig. 37. Stability of somatostatin conjugates **128** and **120** under cytoplasm mimicking conditions

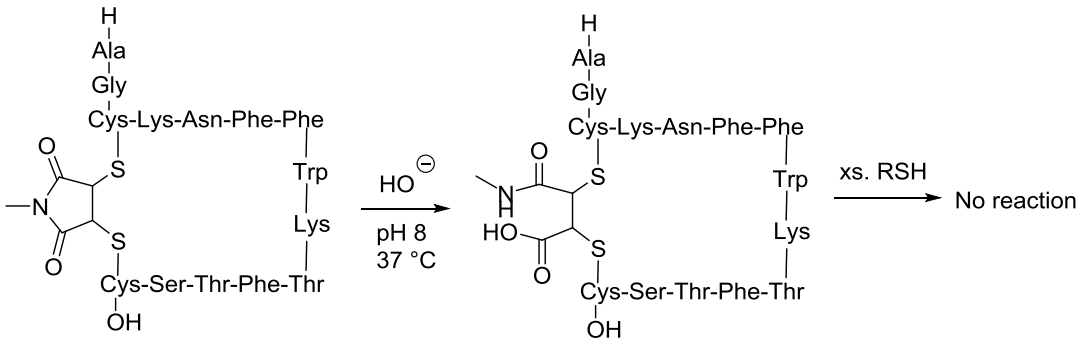
To explain the reactivity of the succinimide bridged construct **128** in the presence of excess thiol, we propose the mechanism depicted in Scheme 105.



Scheme 105. Postulated mechanism for the cleavage of the succinimide bridge in conjugate **128** in the presence of excess thiol

We postulate that conjugate **128** is in equilibrium with the product of the retro-Michael addition, conjugate **173**. The equilibrium is shifted to the side of the succinimide bridged product but in the presence of excess thiol, conjugate **128** will be completely consumed. This is because intermediate **173** reacts with thiols in a conjugate addition type reaction, undergoing cleavage of the maleimide tag. This is in accordance with previous reports within the group.[139]

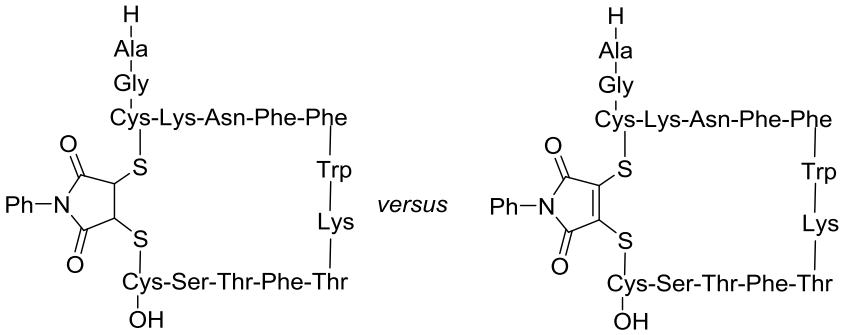
An elegant way to completely inhibit the reaction of the succinimide bridged construct with thiols could be hydrolysis of the succinimide ring (Scheme 106). By hydryolysing the maleimide ring, the pK_a of the α-protons to the carbonyls would increase, preventing the retro-Michael addition which preceeds the loss of the maleimide tag. This hypothesis was inspired by the method developped within our group to render the maleimide bridged conjugates inactive to thiols.[141]



Scheme 106. Strategy for rendering the succinimide bridged constructs thiol stable

Therefore the next step was to investigate ways to achieve controlled hydrolysis of the succinimide ring. For this we decided to focus on *N*-phenyl phenoxymaleimides which are known to be more susceptible to hydrolysis than the *N*-alkyl analogues.[215] Once again, these experiments were performed in parallel with the investigation of the maleimide bridging strategy, so that the two approaches can be more accurately compared.

For this we prepared conjugates **174** and **175,** according to the general procedure for bridging somatostatin with phenoxymaleimides and dibromomaleimides respectively, and then dialysed them into pH 8 phosphate buffer (50 mM sodium phosphate) for 12 h at 0 ^{o}C. The *N*-phenyl dibromomaleimide **176**, necessary for the synthesis of conjugate **175** was prepared according to a literature procedure.[189] The concentration of the peptide conjugates was adjusted to 100 μM and an aliquot taken and analysed by LC-MS. The remaining reaction mixtures were incubated at 37 °C for 1 h and then analysed by LC-MS (Fig. 38). Hydrolysis of both conjugates happens during dialysis. The succinimide bridged conjugate **175** is slightly more stable than the maleimide bridged conjugate **176** but after 1 h at 37 °C both constructs are almost fully hydrolysed. In conclusion, the succinimide bridge has a similar hydrolytic stability to the maleimide bridge and both constructs undergo quick hydrolysis at pH 8.

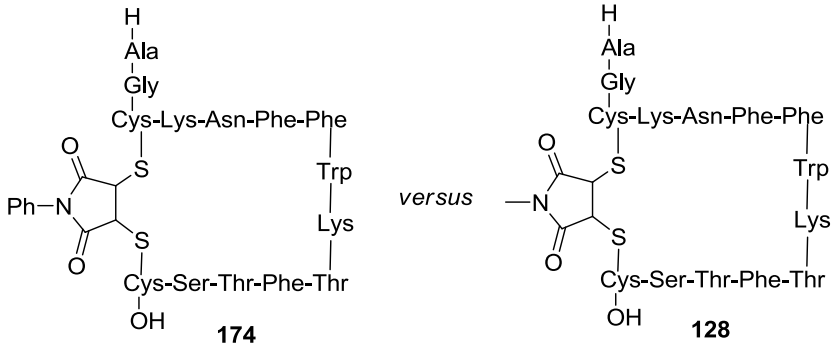


Conjugate	% Hydrolysed	
	after dialysis	after 1 h @ 37 °C
174	75	91
175	92	95

Fig. 38. LC-MS based hydrolytic stabilities of somatostatin conjugates **174** and **175**

The hydrolysed samples of succinimide bridged conjugate **174** and maleimide bridged conjugate **175** were then subjected to a large excess of thiol, 100 eq. of 2-mercaptoethanol and incubated at 37 °C for 21 h. Gratifyingly, LC-MS analysis showed no reaction had occurred.

The influence of the nature of the substituent on the nitrogen atom was assessed by comparing the hydrolytic stability of **174** and **128**. Conjugates **174** and **128** were prepared, according to the general procedures for the succinimide bridging with phenoxymaleimide, and then dialysed into pH 8 phosphate buffer for 12 h at 0 °C. The concentration of both peptide conjugates was adjusted to 100 μM and an aliquot was taken and analysed by LC-MS. The remaining reaction mixtures were incubated at 37 °C for 1 h and then analysed by LC-MS. Fig. 39 shows the percentages of hydrolysed conjugates. The difference between the two conjugates was very significant: while 25% of **174** underwent hydrolysis during dialysis, **128** was fully stable. Even after 1 h of incubation at 37 °C, most of **128** (70%) is present in the reaction mixture while by this time most of **174** (91%) had hydrolysed. However, after 16 h, even the succinimide bridged conjugate **128** is fully hydrolysed.



Conjugate	% Hydrolysed		
	after dialysis	after 1 h @ 37 °C	after 16 h @ 37 °C
174	75	91	98
128	0	30	96

Fig. 39. LC-MS based hydrolytic stabilities of somatostatin constructs **174** and **128**

These results highlight the strength of this approach in tuning the properties of bioconjugates. It was shown that both the nature of the substituent on the nitrogen atom and the nature of the bridge between the cysteine groups can alter the hydrolytic stability of the conjugates. By controlling these parameters, we can choose the optimal strategy for different applications, depending on its requirements.

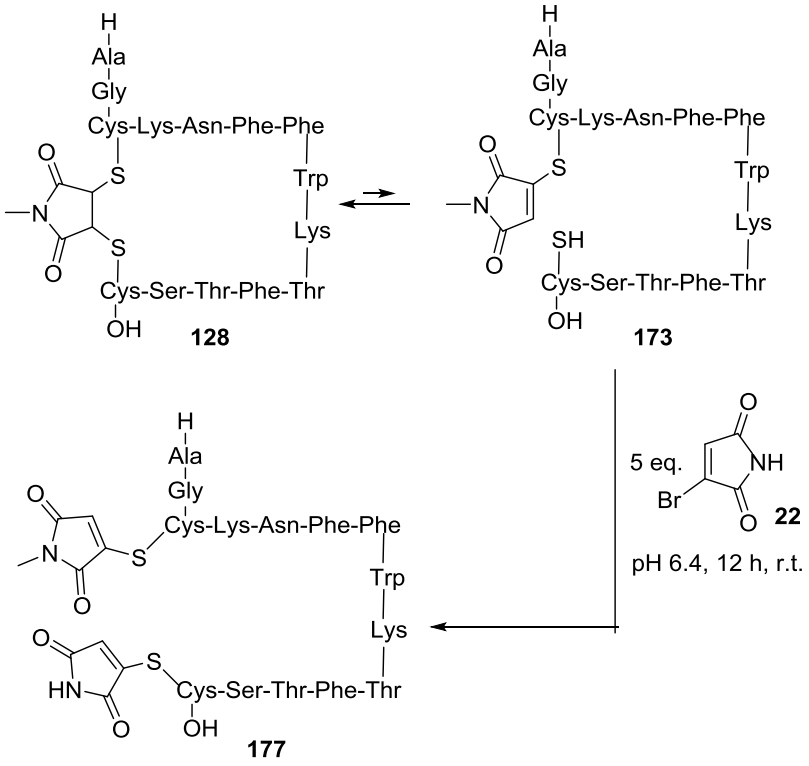
II.5 Differential dual labelling of disulphide bonds

Going back to the mechanism depicted in Scheme 105, we envisaged that the product of the Michael addition, **173** could be trapped with a carefully chosen electrophile. This could be of interest as it could lead to a method to incorporate two labels onto a biomolecule. Such a reagent should display a high reactivity and thiol selectivity. One such electrophile is bromomaleimide.

To test this, conjugate **128** was treated with a slight excess of bromomaleimide **22** (5 eq.) and the resulting mixture was incubated overnight at room temperature (Scheme 107). The different functionalisation on the nitrogen atom of the reagent allows easier interpretation of the LC-MS data. Gratifyingly, LC-MS of the reaction mixture showed one product only, the dual labelled conjugate **177**. At this stage it is important to say that the structure of **177** is based on its mass which indicates that it containes two different maleimide labels, confirming the addition of bromomaleimide **22** to intermediate **173**. It is natural to envisage the

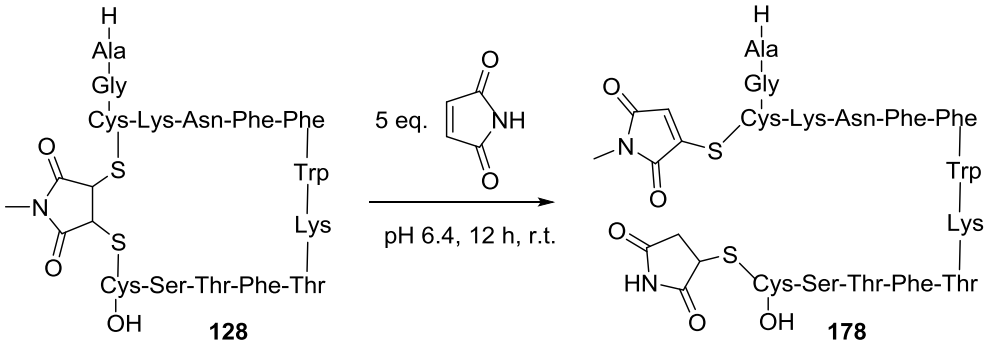
formatio of the two possible regioisomers, as the retro-Michael addition is not expected to be selective. The LC trace offers no indication on the presence of one or two species as there is just one peak that corresponds to the mass of **177**. More details on this will be given later on.

The key species in this transformation is intermediate **173**. The conjugate addition of bromomaleimide on the free thiol in **173** is expected to be the fast step while the formation of **173** is the rate-limiting step. As mentioned before, the equilibrium between **128** and **173** is shifted to the product side due to the constant consumption of **173**. The phosphate ions present in the buffer system act as a base.



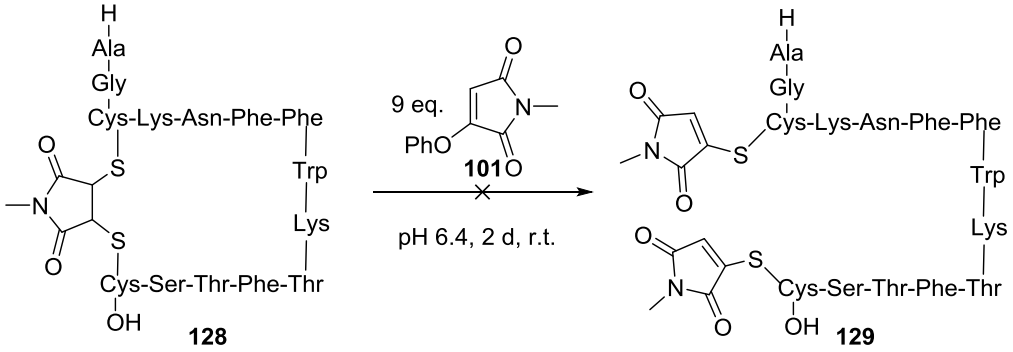
Scheme 107. Dual labelling of somatostatin with bromomaleimide **22**

The transformation was attempted, under the same conditions with another thiol selective electrophile, the commonly employed maleimide. Once again, a sole clean product, **178,** was obtained (Scheme 108). This time, the conjugate contains one maleimide and one succinimide tag.



Scheme 108. Dual labelling of somatostatin with maleimide

The fact that the use of a highly reactive electrophile is critical for the success of the transformation is highlighted by the experiment depicted in Scheme 109. Succinimide bridged somatostatin conjugate **128** is stable, even after 2 days of incubation at room temperature, in the presence of excess of the less electrophilic phenoxymaleimide **101** (9 eq.).



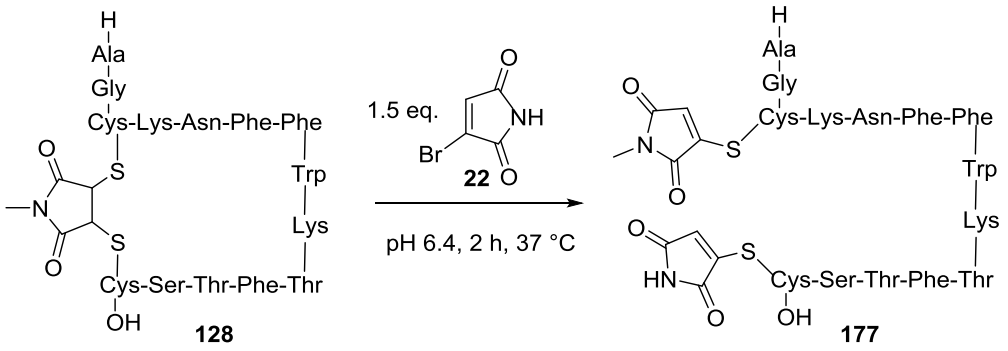
Scheme 109. Stability of conjugate **128** in the presence of excess phenoxymaleimide **101**

This is mainly because the addition of a phenoxymaleimide molecule to intermediate **173** is significantly slower than the intramolecular cyclisation regenerating the succinimide bridged conjugate **128**.

As shown earlier, when a thiol is added to a mixture of bromomaleimide and phenoxymaleimide, the former species is the only one reacting, even in the presence of excess phenoxymaleimide (Fig. 33). This shows potential for developing a one-pot dual modification strategy, without purification between the two steps.

With these results in hand, we set out to optimise the protocol in terms of reaction time and amount of reagent used. We used the reaction between succinimide bridged somatostatin conjugate **128** and bromomaleimide **22** as the model system. A temperature increase was expected to speed up the reaction and therefore it was decided to carry out the reaction at 37 °C. The amount of

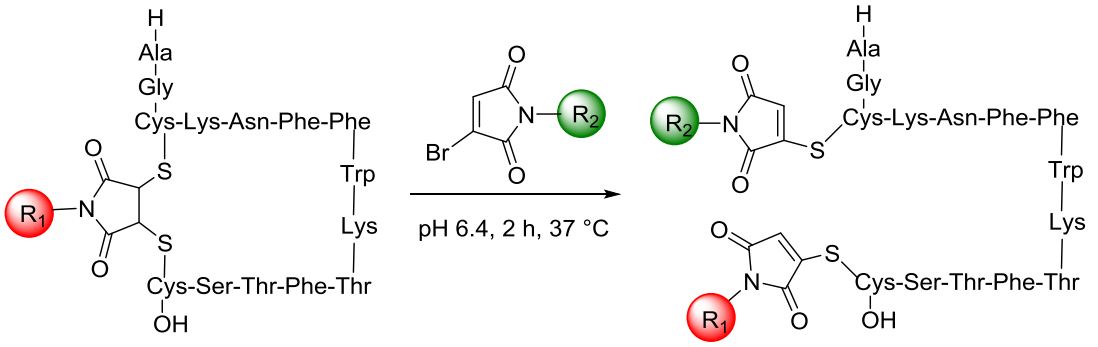
bromomaleimide used vas varied (5 - 1.5 eq.) and gratifyingly, full conversion was achieved within 2 h with only 1.5 eq. of reagent **22** (Scheme 110). The use of the minimum amount of labelling reagent is desired as this is prepared in a DMF stock and a high content of organic solvent can act as a denaturant and alter the conformation of the peptide. No intermediate purification was performed.



Scheme 110. Optimisation of the dual labelling of somatostatin with bromomaleimide **22**

As mentioned in the introduction, methods for the differential dual functionalisation of biomolecules are highly desirable in the areas of proteomics and therapeutics. To show the potential of our approach to access various dual functionalised peptides, we constructed conjugates **177** and **180** - **182**. We highlight here conjugate **182**, bearing two different fluorophores that form a FRET pair and conjugate **180**, bearing a PEG chain and a fluorophore. Labelling with a FRET pair is a commonly employed method to study conformational changes of a protein during folding and unfolding or during binding to another protein or small molecule. PEGylation of a biomolecule also labelled with a fluorophore could prospectively increase the *in vivo* stability of the conjugate which could be useful in imaging studies.

All dual labelled conjugates shown below have been prepared in a single pot starting from already prepared succinimide bridged somatostatin conjugates **128, 161** and **162** (200 µM in pH 6.4 50 mM sodium phosphate containing 40% acetonitrile, 2.5% DMF) (Fig. 40). The succinimide bridged somatostatin was used without purification. The bromomaleimide solutions used were 100x more concentrated than the peptide solution, thus allowing the minimum addition of DMF to the reaction. *N*-Fluorescein bromomaleimide **179** was prepared according to a literature procedure.[177]

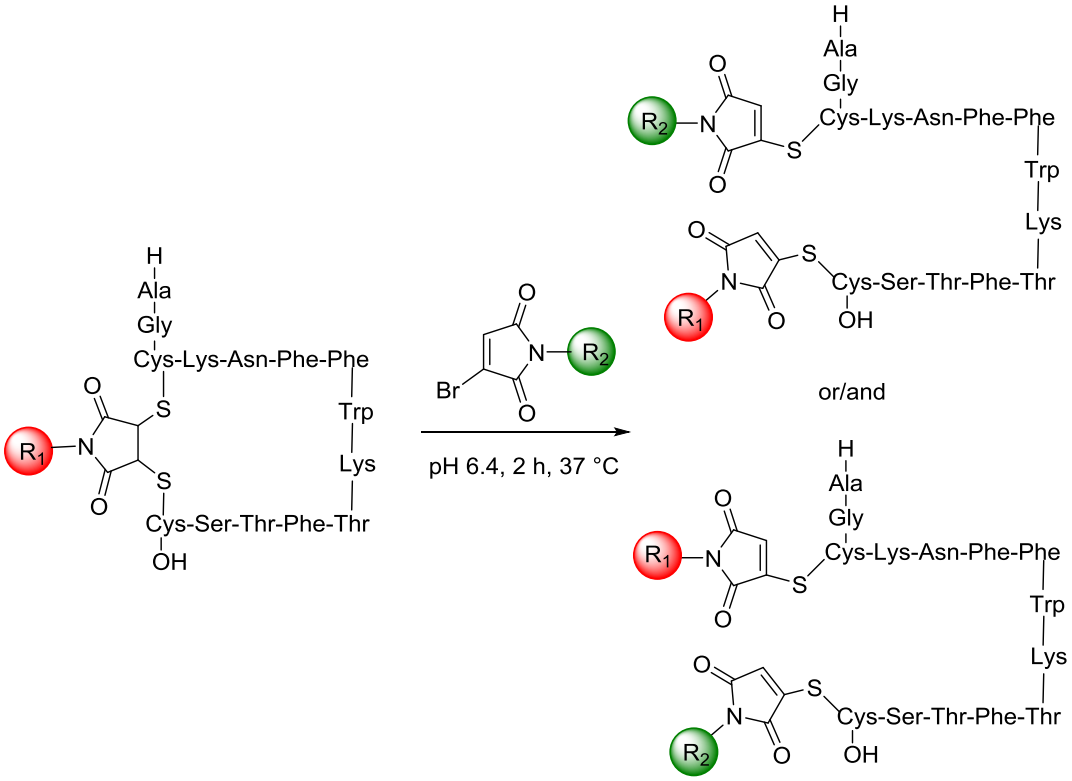


R_1	Conjugate code	R_2	Reagent code	Reagent (eq.)	Product
Me	**128**	H	**22**	1.5	**177**
PEG	**161**	Fluorescein	**179**	4	**180**
PEG	**161**	Me	**57**	3	**181**
Linker-Rhodamine	**162**	Fluorescein	**179**	4	**182**

Fig. 40. Scope of the dual labelling of somatostatin

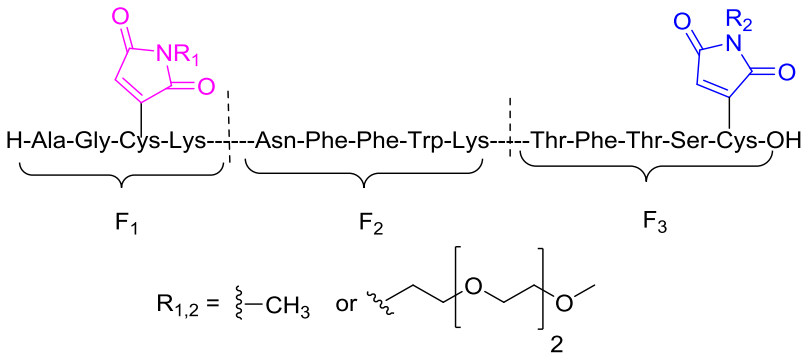
In conclusion, a series of novel bioconjugates of somatostatin were prepared. The protocol is easy, involves minimal amounts of reagents and can be carried out without intermediate purification.

An important issue that remains to be addressed is the regioselectivity of the dual labelling; that is, whether the retro-Michael addition is selective or not. If not, the product of the addition of the second labelling reagent is a mixture of the two possible regioisomers (Scheme 111).



Scheme 111. Possible products of the 2nd step of the dual modification strategy

In order to investigate the selectivity of the Michael addition, dual labelled somatostatin **181** was synthesised and then subjected to trypsin digest. The optimized conditions are 10 : 1 molar ratio between peptide conjugate and trypsin, pH 8.1 buffer (100 mM ammonium acetate, 1 mM calcium chloride) at 37 °C for 15 h. Trypsin is a commonly employed enzyme for peptide mapping that cleaves peptides on the *C*-terminal side of lysine and arginine residues. Looking at the structure of the dual labeled somatostatin conjugate **181**, there will be two points of cleavage, leading to three fragments (F_1, F_2, F_3). Fig. 41 contains the expected masses of the possible products, also taking into account the possibility that the maleimide rings undergo hydrolysis.



R_1	Ring hydrolysed	Mass F_1	R_2	Ring hydrolysed	Mass F_3	Mass F_2
PEG	No	618	PEG	No	799	740
PEG	Yes	637	PEG	Yes	817	
CH_3	No	486	CH_3	No	666	
CH_3	Yes	504	CH_3	Yes	684	

Fig. 41. Possible fragments after trypsin digest of dual labelled conjugate **181**

The mass trace of the peptide sample after digest is shown below (Fig. 42), the retention time for this being 2.54 min, under the same LC-MS conditions described in the Experimental section. The peak corresponding to the middle fragment F_2 is clearly identified ($M+H^+ = 741$). There are also peaks at 799 and 817 which correspond to F_3, labelled with *N*-pegylated maleimide, both non-hydrolysed (M = 799) and hydrolysed (M = 817). Two peaks corresponding to F_1, labelled with *N*-pegylated maleimide, both non-hydrolysed (M = 618) and hydrolysed (M = 637) have been also identified. These peaks have a smaller intensity but this might be due to the difference in the ionisation ability of the three fragments. Peaks with masses corresponding to fragments labelled with the *N*-methyl maleimide have not been detected in the mass trace but the information available indicated the lack of regioselectivity leading to a mixture of the two possible regioisomers as the product of the dual modification strategy. Their ratio is expected to be dependent on the pK_a of the cysteines connected by the succinimide bridge and possibly the steric hindrance induced by the neighbouring amino acids, therefore we can envisage that the regioselectivity varies from protein to protein.

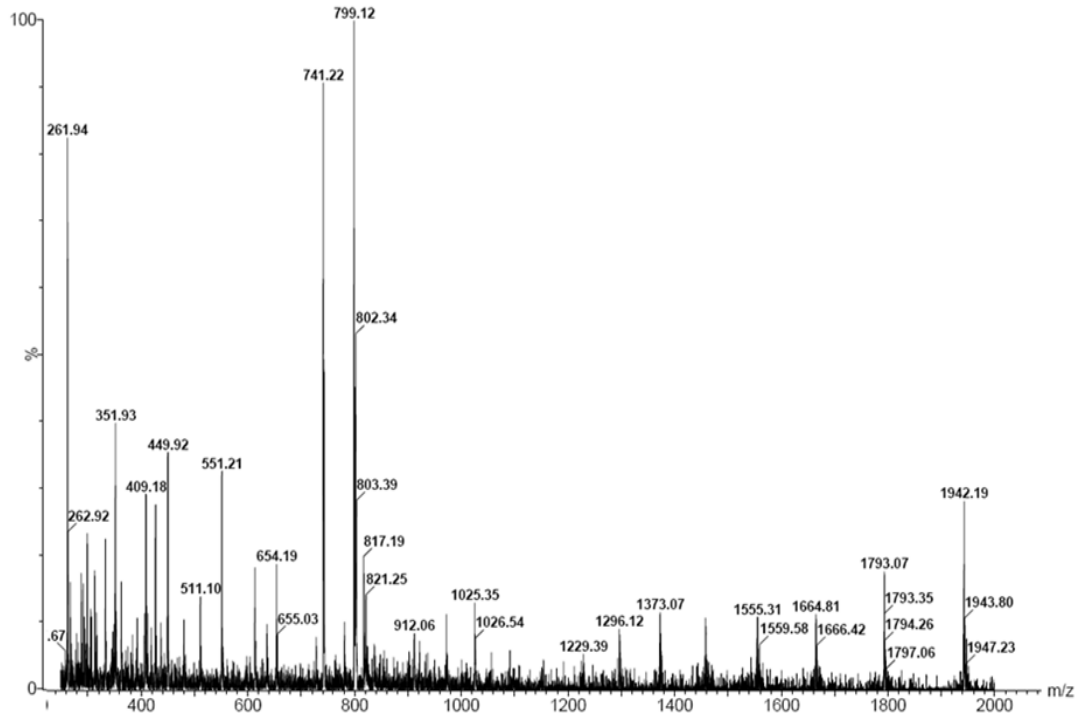
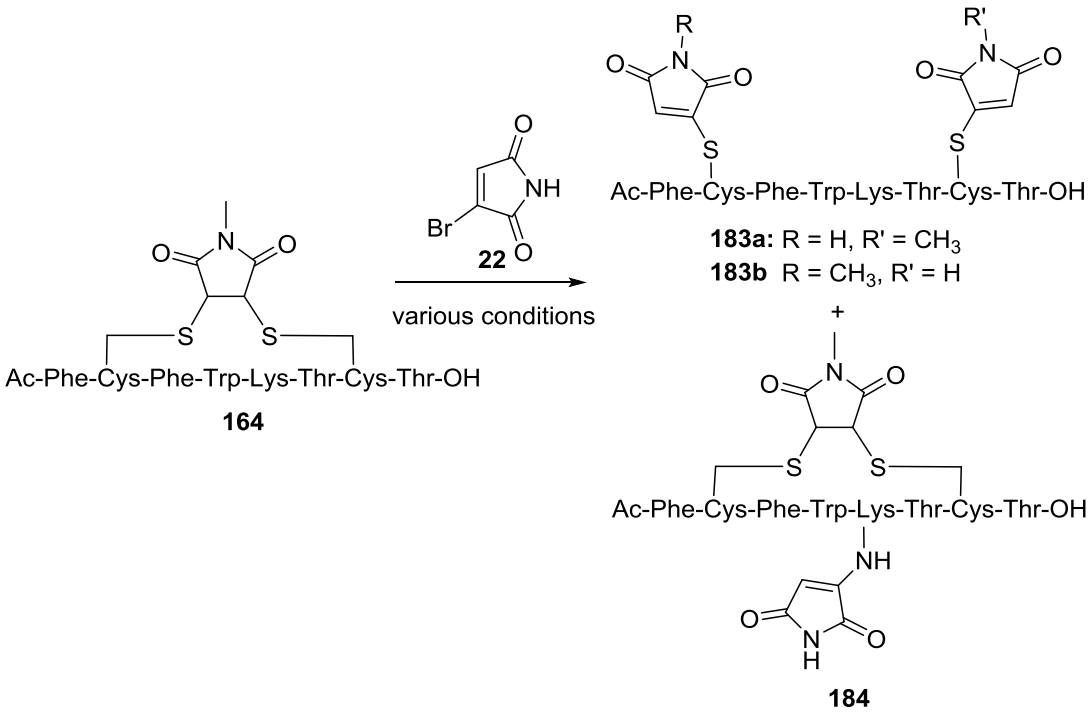


Fig. 42. MS trace of the trypsin digested dual labelled somatostatin conjugate **181**

To illustrate that the methodology is applicable to other peptides we next decided to investigate the peptide octreotide **163**. The dual labelling of the succinimide bridged octreotide conjugate **164** was first attempted using the optimised conditions for somatostatin: 1.5 eq. of bromomaleimide **22** and incubation at 37 °C (Fig. 43, Entry 2). This time, however, LC-MS trace indicated that after 2 h the reaction mixture consisted of unreacted starting material **164** (M = 1130) and two other species. These two species have the same mass (M = 1225) which corresponds to the addition of **22** to the starting material **164** but the significant difference in the retention time indicates a different site of labelling. We postulate that the two products obtained correspond to the structures **183a** and/or **183b** and **184**, and that product **184** is the result of a competing lysine labelling reaction (Fig. 43). This assumption is supported by the fact that the fact that products **183** and **184** have different retention times in the LC trace: **183** was eluted at 1.27 min and **184** at 1.37 min. Another alternative for the side-reaction is the labelling of the *N*-terminus however, this is acetylated therefore unreactive.

An optimisation study was consequently performed, with the aim of achieving full conversion to product **183a** and/or **183b**. The conditions employed are summarised in Fig. 43, together with information on the presence (Y) or absence (N) of each possible product in the reaction mixture.



Entry	22 (eq.)	Temperature (°C)	pH	Time (h)	164	183 (a or b)	184
1	1.5	22	6.4	24	Y	N	N
2	1.5	37	6.4	2	Y	N	N
3	3	37	6.4	2	Y	Y	Y
4	5	37	6.4	1.5	Y	Y	Y
5	5	37	6.4	4	Y	Y	Y
6	5	37	6.4	18	Y	Y	Y
7	5	37	5	18	Y	Y	N
8	5	37	7	18	Y	Y	Y
9	5	37	8	18	Y	Y	Y

Fig. 43. Optimisation study for the dual labelling of octreotide

Unfortunately none of the tested conditions led to a clean product. Full conversion was not achieved, even with 5 eq. of labelling reagent. The side product **184** is formed in considerable amount with respect to the desired product indicating that the two competing reactions - Michael addition at cysteine and lysine - proceed at similar rates. The slower rate of the cysteine modification suggests that the retro-

Michael addition is much slower on octreotide than on somatostatin. As soon as the amount of labelling reagent **22** was increased to 3 eq. the desired product **183** was formed, but unfortunately together with side-product **184**. It is noteworthy to highlight the fact that when the reaction mixture in Entry 3 (Fig. 43) was analysed by LC-MS after 7 days of incubation at room temperature a product that corresponds to the incorporation of three maleimide tags (M = 1320) was observed. We suggest that the structure of product **185** (Fig. 44).

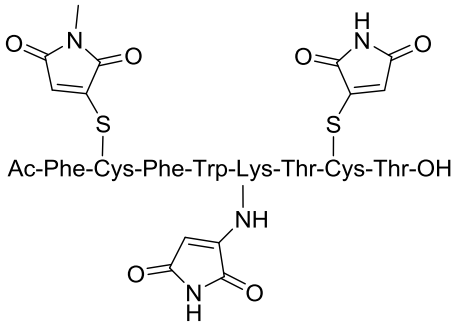


Fig. 44. Postulated structure of side product **185**

The pH screen illustrated the fact that the two competing reactions are influenced in the same way:

(a) a low pH (5) stops the lysines from reacting with bromomaleimide but significantly reduces the rate of the retro-Michael addition; even after 18 h at 37 °C, the conversion was very low, making the protocol not viable;

(b) the product mixture obtained when the reaction was performed at pH 7 was very similar to the one obtained at pH 6; in both cases, the two products are obtained in comparable amounts, together with unreacted starting material;

(c) an increase in the pH (8) speeds up both reactions, therefore the ratio between the two products is preserved. In this case, though, hydrolysis of the maleimides rings also takes place and this completely inhibits the retro-Michael addition, leading to an overall decrease of the percentage of desired product in the reaction mixture.

With no other solutions in hand for stopping the labelling reagent from reacting with the lysine group on octreotide, we decided to try different labelling reagents that would hopefully display a higher selectivity for thiols compared to lysines. Dr. Vijay Chudasama in the Caddick group at UCL reported on the use of substituted pyridazinediones **186** and **187** for cysteine labelling and some available analogues were tested.[216] Another reagent investigated by Dr. Chudasama was the amide **188**, which was shown to act as a low reactivity conjugate acceptor with high

selectivity for thiols. We also tested maleimide (Fig. 45). All reactions have been performed at in pH 6.4, 50 mM phosphate buffer containing 40% CH_3CN and 2.5% DMF at 37 °C.

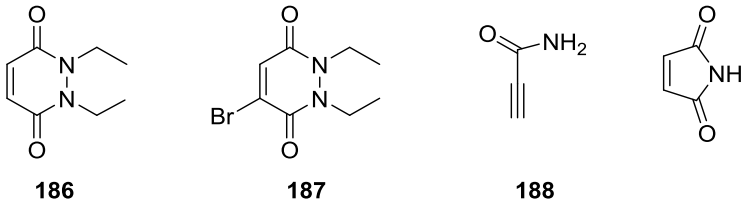
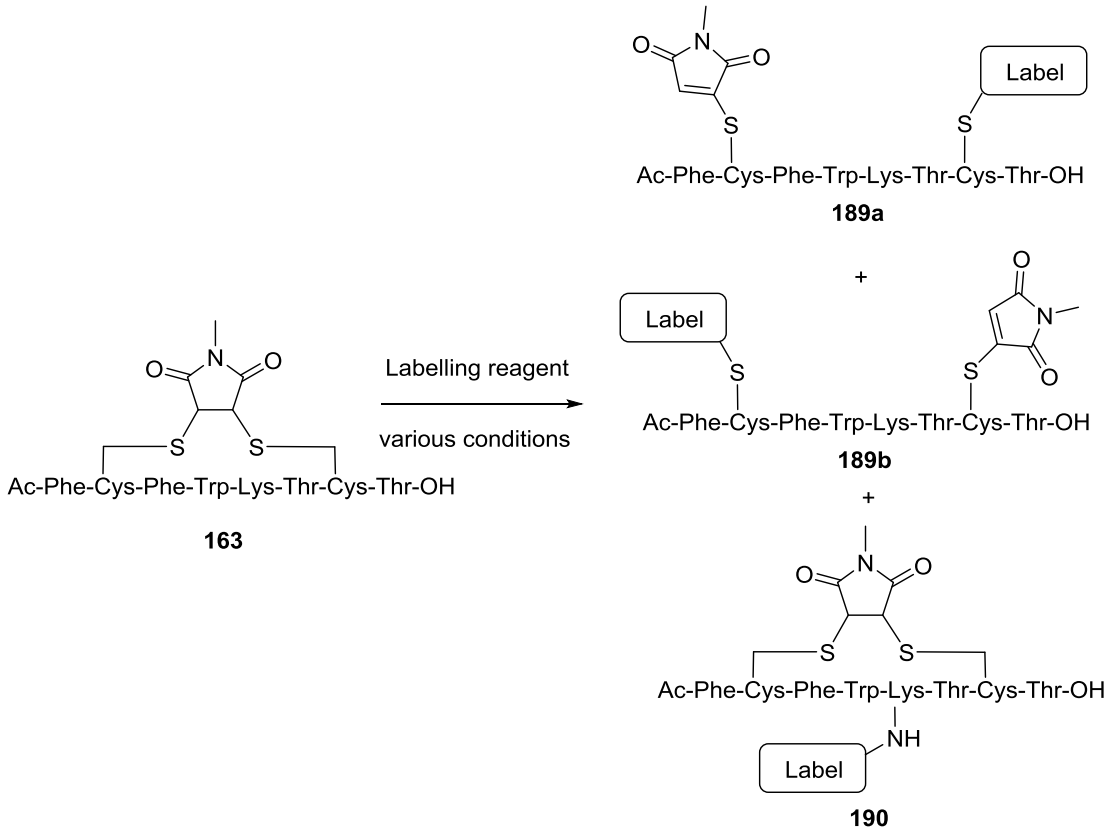


Fig. 45. Structures of cysteine labelling reagents tested

Unfortunately, all of the reagents tested failed to satisfy our requirements; in all cases when a reaction occurred (Entries 1, 7 and 8, Fig.46) the desired product **189a** or/and **189b** was obtained together with the lysine modified product **190**. Except for maleimide, which is known to be highly reactive, the other reagents displayed significantly lower reactivity so that high amounts of reagents (100 - 300 eq.) were necessary to promote the reaction.



Entry	Reagent (eq.)	Time (h)	163	189	190
1	Maleimide (5)	2	Y	Y	Y
2	**186** (1.5)	2	Y	N	N
3	**187** (1.5)	2	Y	N	N
4	**188** (1.5)	2	Y	N	N
5	**188** (200)	19	Y	N	N
6	**187** (100)	19	Y	Y	N
7	**187** (100)	130	Y	Y	Y
8	**187** (300)	72	Y	Y	Y

Fig. 46. Scope of the dual labelling of octreotide

Lastly, we investigated the utility of using phenoxymaleimide **101**, as this is less reactive than bromomaleimide and maleimide and more reactive than the pyridazinediones shown in Fig. 47**.** For this, PEG-succinimide bridged octreotide conjugate **191** was treated with *N*-methyl phenoxymaleimide **101** (15 eq.) and the reaction mixture was incubated at 37 °C for 30 h. The mass trace of the reaction

mixture at the end of the reaction indicated that the conversion of the reaction was unsatisfactory and that in addition to unreacted starting material **191** (eluted at 2.74 min, M = 1263), the product mixture contained both the desired product **192** (eluted at 2.38 min, M = 1371) and the lysine modified product **193** (eluted at 3.12 min, M = 1371).

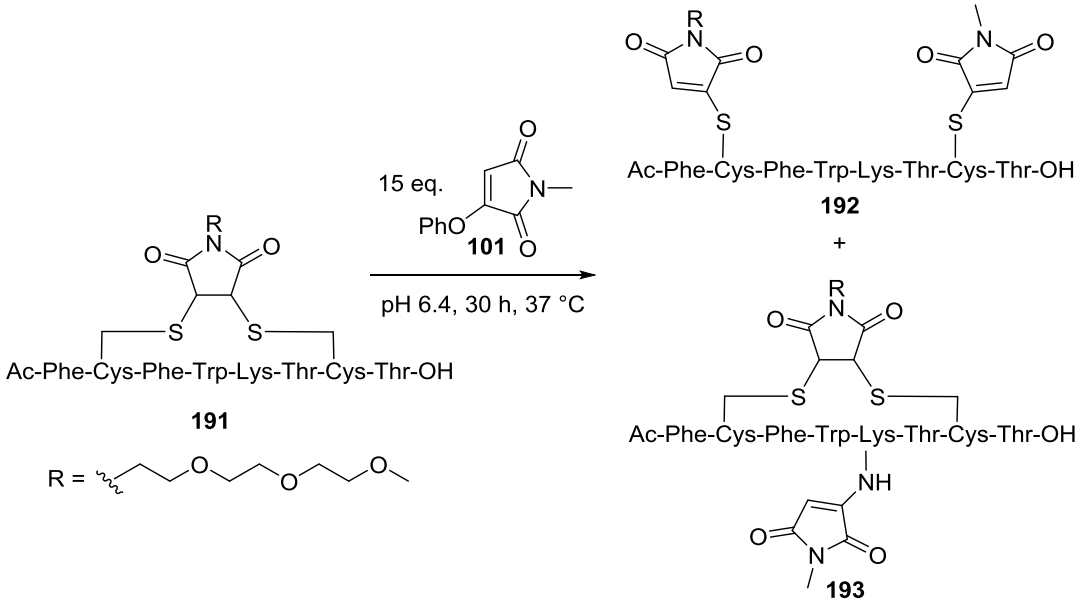
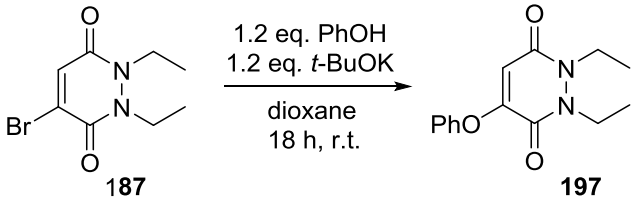


Fig. 47. 2nd step of dual labelling of octreotide using phenoxymaleimide **101**

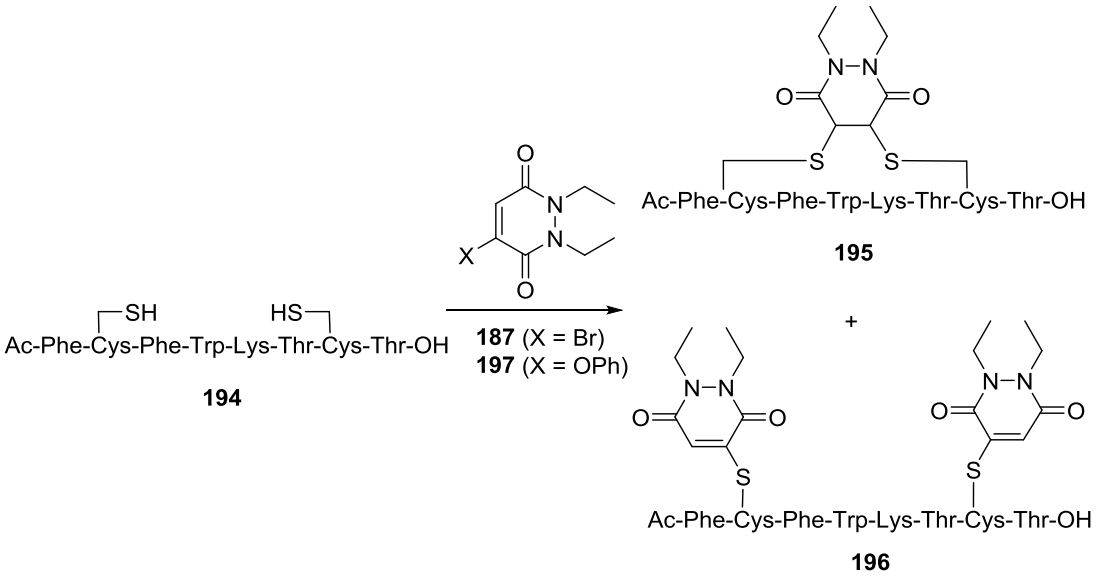
Another strategy was to use the bromopyridazinedione to create a 6-membered saturated ring bridge, similar to how phenoxymaleimide was used. In this way the pK_a of the α-protons next to the carbonyl groups in the ring would be altered, leading to a change in the rate of the retro-Michael addition. Various amounts (1.5 - 10 eq.) of bromopyridazinedione **187** were added to reduced octreotide **194** (200 µM in pH 6.4 50 mM phosphate buffer, 40% CH_3CN, 2.5% DMF) and the reaction was monitored by LC-MS over 1 h (Fig. 48, Entries 1-3). The reaction was maintained at room temperature. Unfortunately, all attempts to obtain a clean bridged octreotide conjugate failed. The reaction either did not proceed or gave a mixture of the desired bridged peptide conjugate **195** (M = 1187) and the *bis*-labelled product **196** (M = 1356). This was surprising given the fact that previous data indicated that bromopyridazinedione is less reactive than the phenoxymaleimide. It seems that the rate of addition of the second molecule of labelling reagent **187** is of the same order of magnitude as the intramolecular cyclisation leading to the desired product. An even less reactive labelling reagent was needed, and in analogy with the scale of reactivity between bromo and phenoxymaleimides, a novel pyridazinedione analogue was designed:

phenoxypyridazinedione **197** (Scheme 112). This was obtained by conjugate addition of phenol to bromopyridazinedione **187**, under the optimised conditions for the addition of phenol to bromomaleimide (Chapter II.1).



Scheme 112. Synthesis of phenoxypyridazinedione **197**

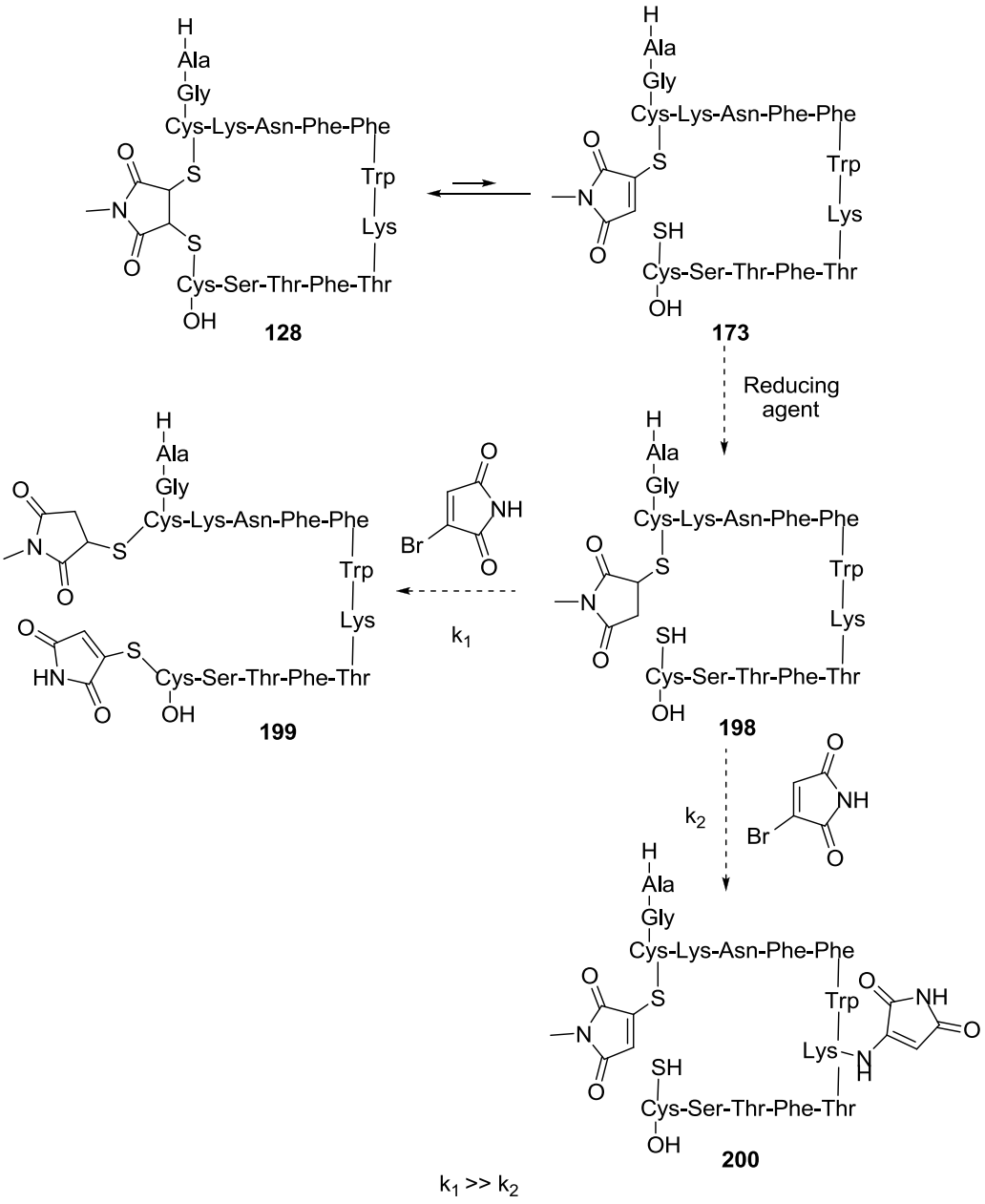
Indeed, this reagent was significantly less reactive. Quantities as high as 500 eq. and a temperature of 37 °C were employed to achieve full conversion of the reduced octreotide **194** but unfortunately the product mixture contained both the mono-labelled **195** (M = 1188) and the *bis*-labelled peptide **196** (M = 1356) conjugates (Fig. 48, Entry 5-6).



Entry	X	Reagent (eq.)	Temperature (°C)	Time (h)	194	195	196
1	Br	**187** (1.5)	22	1	Y	N	N
2	Br	**18**7 (5)	22	1	Y	Y	Y
3	Br	**187** (10)	22	1	Y	Y	Y
4	OPh	**197** (5)	22	24	Y	N	N
5	OPh	**197** (100)	37	23	Y	Y	Y
6	OPh	**197** (500)	37	23	N	Y	Y

Fig. 48. Scope of the addition of substituted pyridazine diones to reduced octreotide

Another strategy that was investigated for tackling the competing lysine modification is depicted in Scheme 113. This new approach focuses on the retro-Michael addition product **173** rather than on optimising the conditions of the second step or the labelling reagent used. We postulate that by reducing the maleimide in **173** to the corresponding succinimide, the intramolecular cyclisation will be inhibited, making the thiol fully available for the reaction with the labelling reagent (e.g. bromomaleimide). This thiol labelling is expected to be far faster than the lysine modification, ensuring the formation of the desired dual labelled product **199** in high yields.

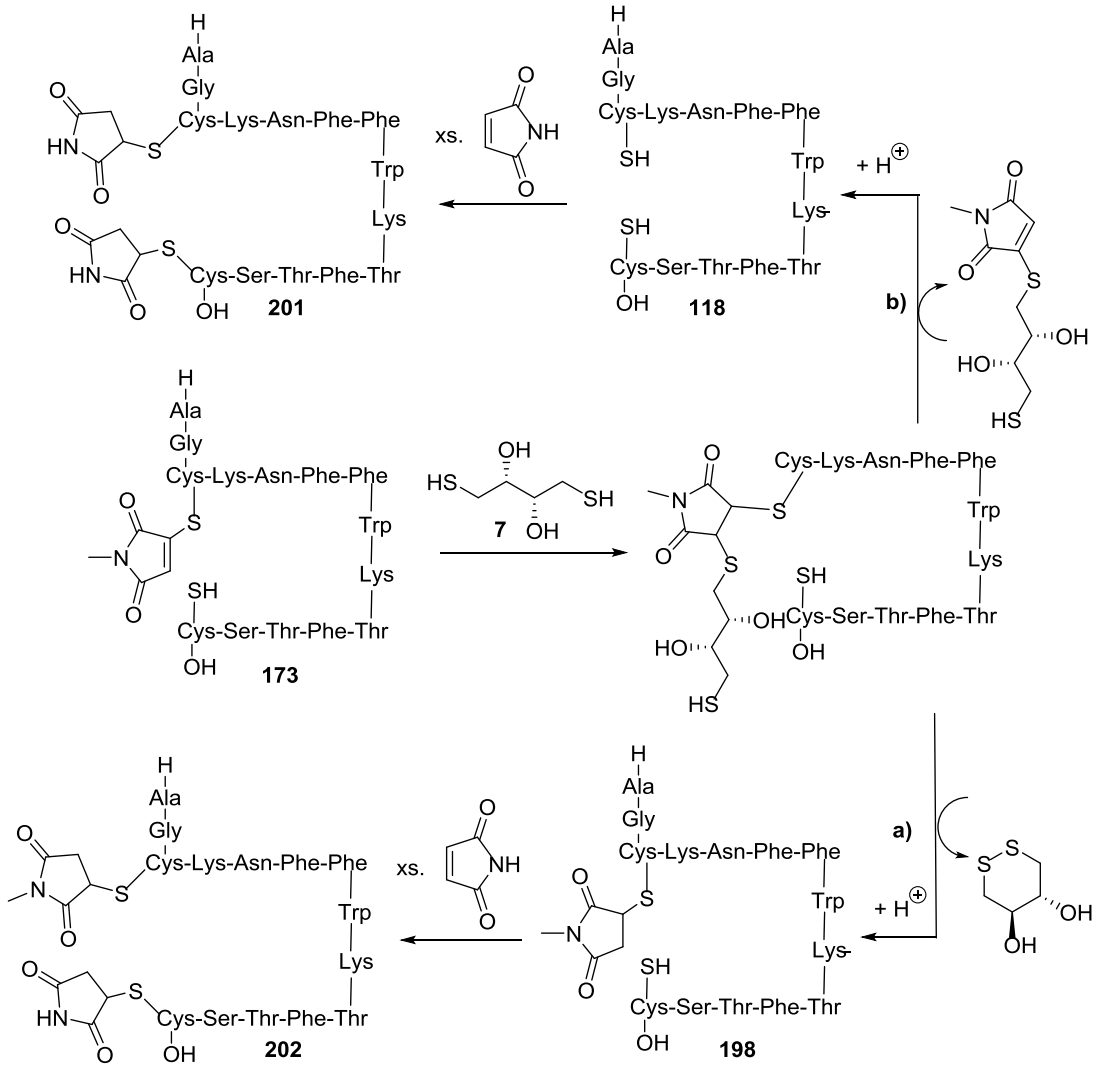


Scheme 113. Strategy for inhibiting the lysine modification side-reaction

As it was readily available, somatostatin **29** was used as a model system and the reducing agents investigated were dithiothreitol and TCEP.

Firstly DTT **7** was investigated. As depicted in Scheme 114, when DTT **7** is added to the succinimide bridged somatostatin **128**, the reducing agent is expected to react with the product of the retro-Michael addition reaction, **173** by attacking the double bond in the maleimide ring. The next step would consist of the cyclisation of DTT **7** to give the 6-membered ring containing an internal disulphide bond and an intermediate enolate that could either abstract a proton to give product **198** (Scheme114, route a) or undergo cleavage of the maleimide tag to give reduced

somatostatin **118** (Scheme 114, route b). The addition of excess maleimide is a simple and efficient way of quantifying the products present in the product mixture.



Scheme 114. Possible reaction routes for the addition of DTT to x

A series of DTT **7** concentrations (10 - 100 eq.) were screened. The reaction mixtures were incubated for 2 h at 37 °C and the results are shown in Table 4.

DTT 7 (eq.)	Temperature (°C)	Time (h)	128 (%)	201 (%)	202 (%)
10	37	2	18	47	28
25	37	2	12	65	23
50	37	2	11	63	26
100	37	2	0	72	28

Table 4. Testing the reduction of **173** with DTT **7** (10 - 100 eq.)

The major product was in all cases **201**, the product derived from reduced somatostatin **118** (M = 1834) (Scheme 115, route b). The desired product **202** (M = 1849) was also formed but as the minor product. Furthermore, the higher the concentration of DTT, the more cleavage occurs. We then decided to investigate the reactions over a shorter period of time (15 min and 30 min time points) using a small excess of reducing agent (5 and 10 eq.), (Table 5). Unfortunately, the major product in this case is the unreacted starting material **128** (M = 1750). Both products **201** and **202** appear to form under these reaction conditions and it seems difficult to favour the formation of one over the other.

DTT 7 (eq.)	Temperature (°C)	Time (min)	128 (%)	201 (%)	202 (%)
5	37	15	62	13	25
	37	30	56	25	19
10	37	15	66	10	24
	37	30	60	20	20

Table 5. Testing the reduction of **173** with DTT (5 and 10 eq.)

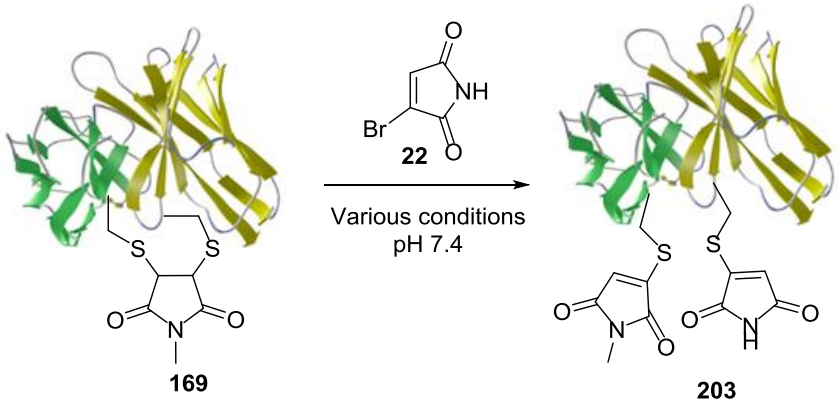
The use of TCEP was also investigated. Excess TCEP (65 eq.) was added to a sample of succinimide bridged somatostatin **128** and then incubated at room temperature for 6 h. According to the same principle as the previous experiment, maleimide (100 eq.) was added to quench the reaction. LC-MS analysis of the final reaction mixture indicated that no reaction had occurred.

In conclusion, efforts to prevent the lysine labelling during the second step of the dual modification of octreotide by reducing the maleimide ring in intermediate **173** proved unsuccessful. The competing lysine labelling seems to be favoured by the same factors as the retro-Michael addition (increased temperature, pH, increased reactivity of the labelling agents) therefore a new approach should be found to inhibit it.

To explore the scope of the dual labelling with respect to the protein substrate, we decided to test it on a larger protein. The ds-scFv **33** was the model system of choice as the disulphide bond is artificially inserted therefore not essential for retention of the biological activity of the protein.

The temperature (22 °C or 37 °C) and the amount of the bromomaleimide **22** (5 - 50 eq.) were varied in order to find the optimal reaction conditions to produce the highest conversion to a product whose mass corresponds to structure **203** (Fig.

49). It is important to highlight that the selectivity of the retro-Michael addition has not been investigated in the case of the ds-scFv substrate therefore the identity of product **203** in terms of regioselectivity is not confirmed. All reactions were performed after the ds-scFv succinimide bridged conjugate **169** was separated from the small molecules in the first step (reducing agent and labelling reagent) by ultracentrifugation and after the concentration was readjusted to 70 µM.



Entry	22 (eq.)	Temperature (°C)	Time (h)	Comments on the product mixture
1	20	22	24	Unreacted **169** (M = 26850), part of a complex mixture
2	5	37	2	Unreacted **169** (major product) + **203** (M = 26945) + unidentified species (M = 27010)
3	10	37	5.5	Main products: species with M = 26992 (2 maleimides, 1 hydrolysed + Na), another species with M = 27115 (3 maleimides, 2 rings hydrolysed + Na), part of a complex mixture
4	50	37	2.5	**203** (M = 26945) is the main product but still some unreacted **169** (M = 26850) + other minor unidentified products
5	50	37	3.5	Complex mixture, contains degradation products

Fig. 49. Optimisation of the 2nd step of the dual labelling of ds-scFv conjugate **169**

When the transformation was performed at room temperature and 20 eq. of bromomaleimide **22** were used, no reaction occurred even after 24 h. This indicated that the reaction is much slower than in the case of octreotide and somatostatin, so it was decided to raise the temperature to 37 °C. When 5 eq. of bromomaleimide **22** were employed, the reaction mixture after 2 h consisted of unreacted starting material **169** (M = 26850), a species whose mass (M = 26945) corresponds to that

of the desired product **203** and an unidentified species (M = 27010). It was then decided to use more bromomaleimide **22** (10 eq.) and leave the reaction for longer (5.5 h) but in this case the LC-MS showed a complex mixture, indicating a significant amount of degradation. We postulate that in this case the maleimide rings undergo hydrolysis, leading to product **204** (M = 26992) and also that lysine modification takes place, leading to a product **205** whose mass (M = 27115) corresponds to the incorporation of three maleimide rings, two of them showing as the Na carboxylate salts (Fig. 50). It is important to highlight the fact that the site of hydrolysis is not determined therefore the structures below should not be taken as definite; the structures for **204** and **205** therefore only correspond to one of the possible isomers.

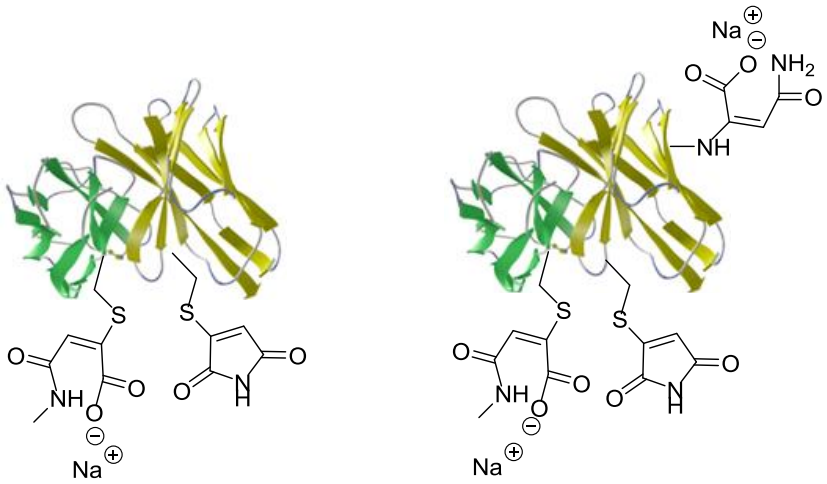
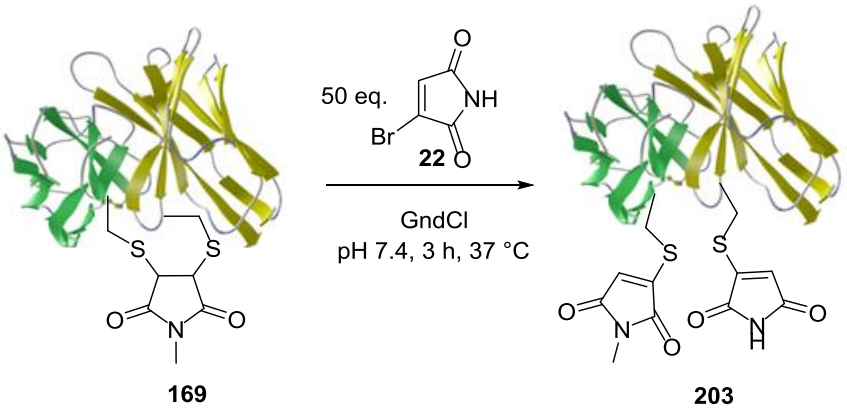


Fig. 50. Postulated structures for the products in Fig. 49

The difficulty in achieving the required transformation is correlated to the fact that the disulphide bond in this protein is quite buried in the tertiary structure and therefore it is less prone to react. To test this hypothesis, we decided to investigate the use of a denaturant, in our case guanidinium chloride. Denaturants are chemicals that interfere with the intramolecular interactions mediated by non-covalent forces (i.e. hydrogen bonds, van der Waals forces, and hydrophobic effects) in the protein.[217] At 6 M GndCl concentration all proteins are completely denatured (and hence unfolded). Our interest was in achieving a partial unfolding which would expose the disulphide of interest, so we screened several concentrations of GndCl.

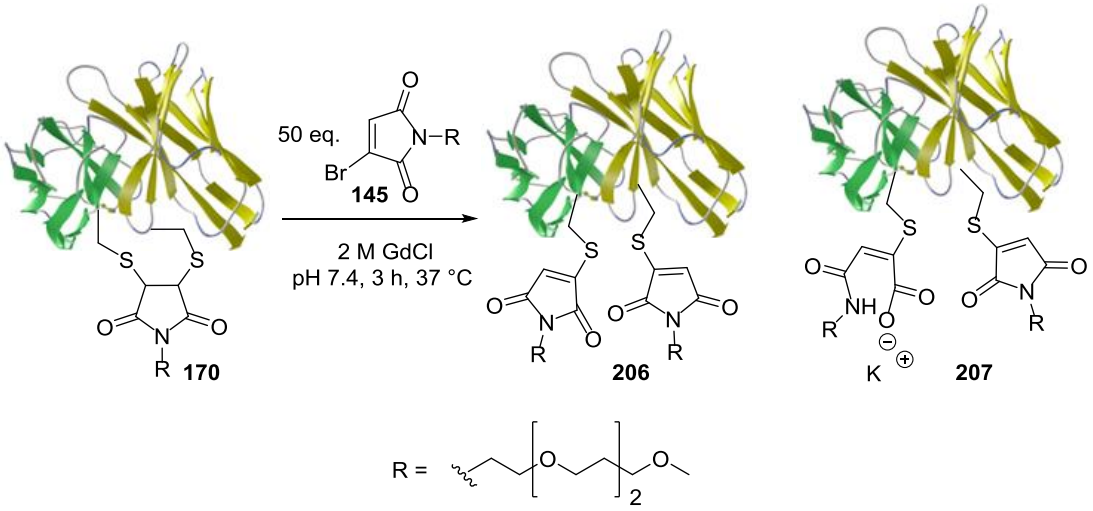


GndCl (M)	Comments on the product mixture
0	Complex mixture (mostly unreacted **169**)
1	Complex mixture (**203** together with unidentified species with masses M = 27015 and M = 27106)
2	**203** is the sole product
3	Complex mixture (**203** together with **33**, unidentified species M = 27358 and M = 28609 and degradation products)

Fig. 51. Effect of guanidinium chloride addition on the 2nd step of the dual labelling of ds-scFv conjugate **169**

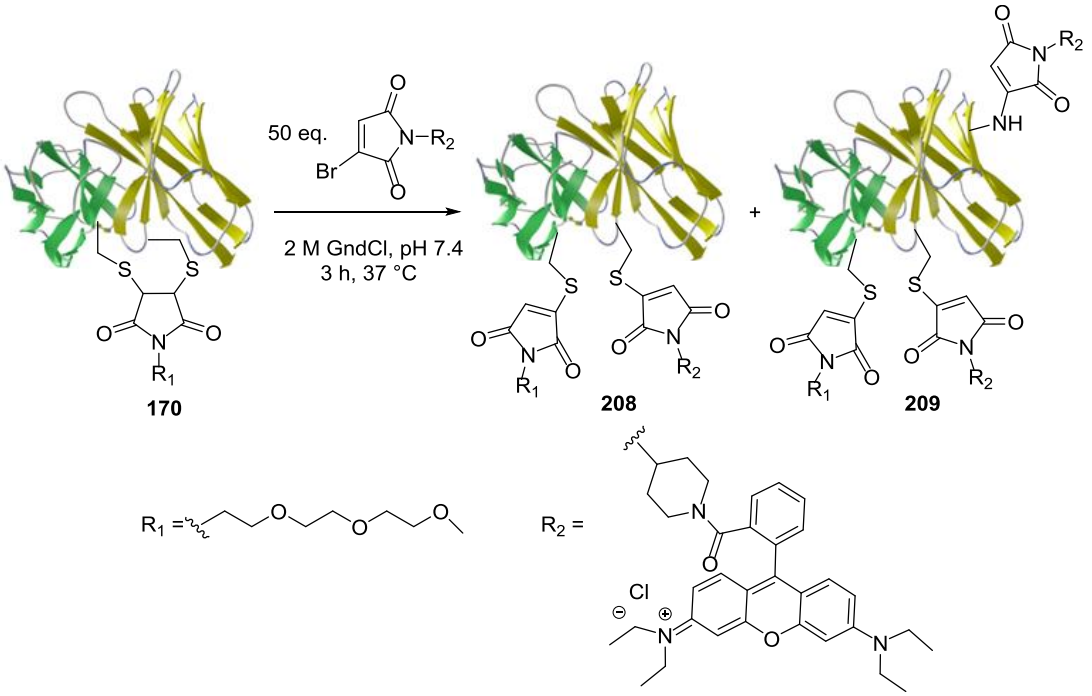
The cleanest reaction mixture was obtained at 2 M GndCl, after 3 h of incubation at 37 °C and with 50 eq. bromomaleimide **22** (Fig. 51). The major peak in the LC-MS spectrum corresponds to the *bis*-labelled ds-scFv conjugate **203** (M = 26949).

We then applied these optimised conditions to the reactions depicted in Schemes 116 and 117. In both cases the conversion was 100% but the formation of the desired product was accompanied by unwanted side-reactions. When the *bis*-PEG-ylation was attempted, a species with a mass of 39 units higher than the one of the *bis*-labelled product was observed (Scheme 115). We suspect that one of the maleimide rings hydrolysed to give a 7 : 3 mixture of product **206** and **207**, based on the LC-MS trace. The site of hydrolysis could not be determined, therefore the structure proposed for compound **207** is only of one of the two possible isomers. Additionally, there is no information whether compound 206 was obtained as a single regioisomer or together with its regioisomer, with the maleimide rings with swapped positions. For simplicity, only one structure is shown.



Scheme 115. 2nd step of the dual labelling of ds-scFv using *N*-PEG bromomaleimide **145**

When 50 eq. of *N*-Rhodamine bromomaleimide **147** were added to the succinimide bridged conjugate **170**, a product with a mass corresponding to the addition of two *N*-Rhodamine maleimide tags was obtained in a 4 : 6 ratio together with the desired product **208** (Scheme 116). No information on the regioselectivity of the retro-Michael addition is available therefore the possibility of conjugate **208** existing as a mixture of the two possible regioisomers should not be neglected. Based on the results obtained when investigating the reactivity of octreotide, we postulate that the side product could be the result of lysine-modification and therefore corresponds to product **209**.



Scheme 116. 2nd step of the dual labelling of ds-scFv using *N*-Rhodamine bromomaleimide **147**

In conclusion, the dual labelling strategy of ds-scFv **33** suffers from the same limitations as when octreotide **163** was investigated: slow kinetics of the retro-Michael addition requiring excess labelling reagent, increased temperature and addition of a denaturant (only in the case of ds-scFv) which favour the addition of the labelling reagent to a lysine residue. In all these transformations, the optimised protocol led to the desired dual labelled product as the major product hence the strategy could still be used, provided the lysine-modified side-product can be separated. The success of the dual labelling depends on the accessibility of the disulphide and on how essential it is in the tertiary structure of the protein. The effect on the protein activity is in fact another aspect that needs to be considered when choosing the labelling strategy. In our case, the disulphide bond remains broken, therefore activity of the protein could be severely altered. We envisage that our approach could be most useful for the labelling of engineered disulphides. In this instance, they could also be designed to be solvent accessible and near aminoacids residues that would stabilize the resulting thiolate, hence potentially favouring the Michael addition.[218]

III Conclusions

In conclusion, the present work details the synthesis of a library of novel mono- and disubstituted maleimides (~30 analogues) with the aim of establishing the influence of the substituent(s) on the double bond on the reactivity of these reagents with cysteine and also developing reagents with tuned properties. A faster, more reliable and suitable for scale-up synthesis of bromomaleimide was established. The rest of the analogues were obtained mainly *via* conjugate addition reactions with appropriately chosen nucleophiles starting from bromomaleimide or the commercially available dibromomaleimide. Sulfoxymaleimides were obtained *via* oxidation of the corresponding thiomaleimides. Various attempts to further oxidize the sulfoxymaleimides to the sulfonylmaleimides were unsuccessful.

The utility of the novel monosubstitued analogues as protein labelling reagents was then tested on a model protein- the single cysteine mutant of the Grb2 SH2 domain protein.

It was shown that by varying the leaving groups on the maleimide core, a range of novel cysteine labelling reagents could be produced with tuneable properties (Fig. 52). Among the monosubstituted analogues, sulfoxymaleimides are the most reactive however, excess reagent is required due to their degradation in aqueous systems. The halomaleimides are all very fast and efficient labelling reagents, with similar reactivity profiles. At the other end of the spectrum, phenoxymaleimides display reduced reactivity. Fine tuning could be achieved by installing different groups on the phenyl ring. The main advantages of phenoxymaleimides are their ideal compatibility with the *in situ* protocol and the fact that the reagent can be rendered water soluble by installing a short PEG chain on the phenyl ring. Methoxymaleimides are even less reactive compared to phenoxymaleimides and lead to a mixture of the protein thiomaleimide conjugates as the minor product and the the protein methoxythiosuccinimide as the major product. Aminomaleimides proved unreactive towards the cysteine in the Grb2 SH2 (L111C), despite using excess reagent.

Additionally, a kinetic study was designed in order to provide a more quantitative comparison between the reactivities of halomaleimides and cysteine. Glutathione addition to chloro-, bromo-, iodo- and phenoxymaleimide, leading to thiomaleimide **126** was monitored by UV-spectroscopy, based on the absorption of the thiomaleimide product.

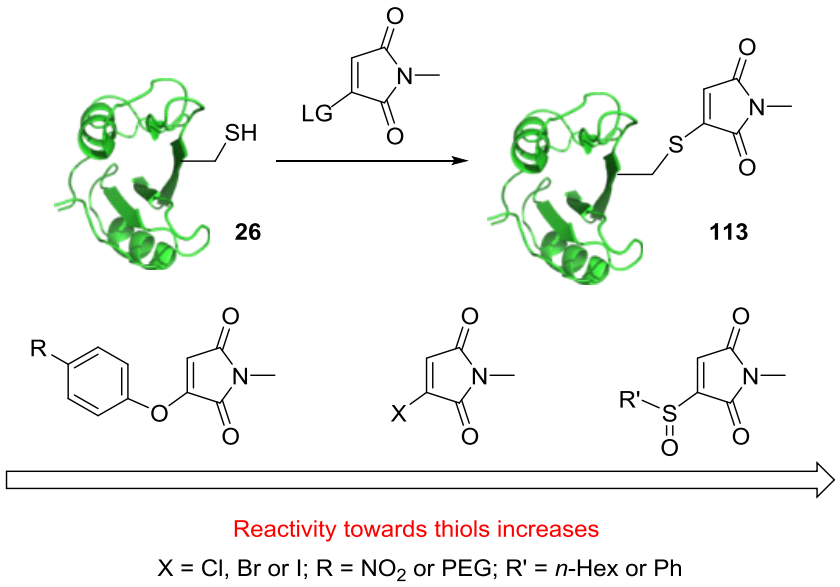
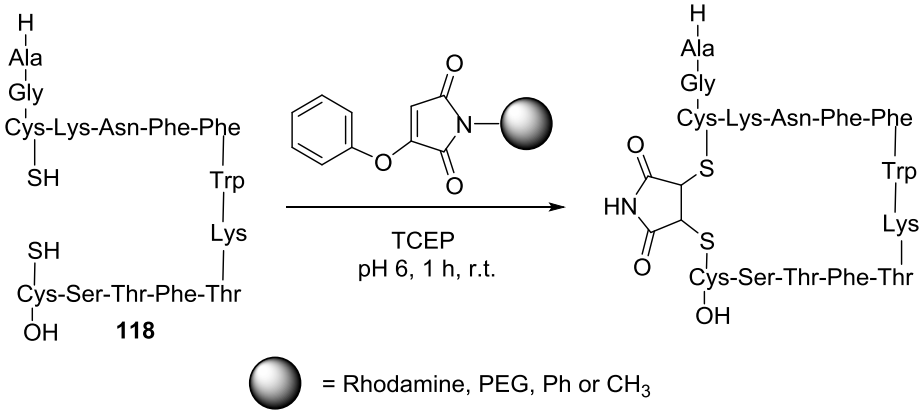


Fig. 52. Reactivity scale of selected monosubstituted maleimides

When reacted with reduced somatostatin **118**, phenoxymaleimides led to the succinimide bridged product only as opposed to bromomaleimide which led to a mixture of a *bis*-addiction product and the succinimide bridged product (Scheme 117). This enables phenoxymaleimides to be employed in succinimide bridging, offering a novel strategy for labelling of disulphide containing biomolecules (shown on two peptides (somatostatin **26** and octreotide **163**) and three antibody fragments (ds-scFv 33, Rituximab Fab **165** and Trantuzumab Fab **167**). This strategy is fast, efficient and perfectly compatible with the *in situ* protocol. The use of the water soluble phenoxymaleimide synthesized allowed the transformation to occur under organic solvent free conditions.

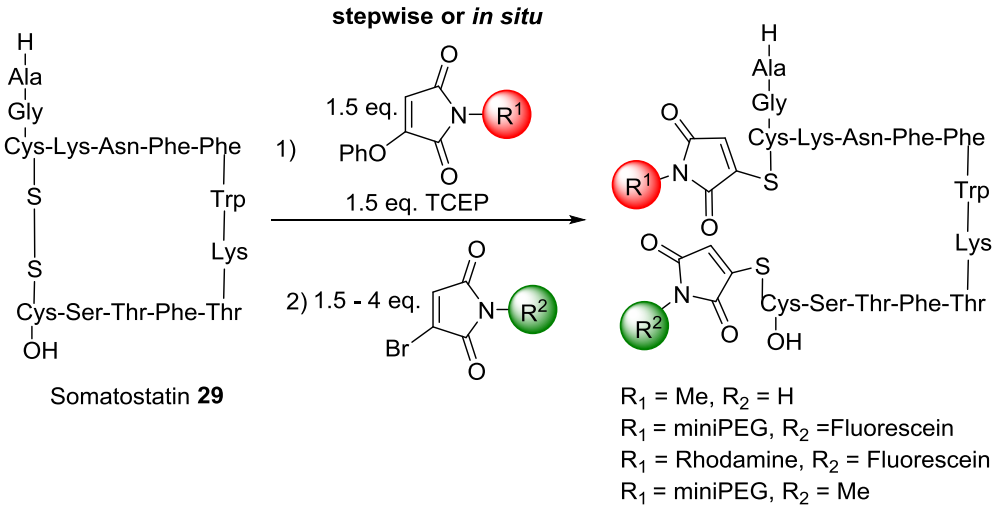


Scheme 117. Phenoxymaleimide addition to reduced somatostatin

Furthermore, the succinimide bridge was shown to be reversible and that the intermediate could be trapped with a suitable chosen electrophile such as

bromomaleimide or maleimide. This discovery led to the development of a one-pot dual labelling strategy of both peptides and proteins. The dual labelling works well on somatostatin, as supported by various dual labelled somatostatin conjugates prepared (Scheme 118). It was shown using somatostatin as substrate, that the retro-Michael addition is not regioselective and that in fact, the dual modified products are mixture of the two possible regioisomers. For the sake of simplicity, Scheme 118 shows only one of the two possible regioisomers.

The strategy was also tested on the ds-scFv **33**, for which an optimized protocol was established involving addition of 2 M guanidinium chloride to partially unfold the protein and therefore enhance the reactivity of the disulphide by increasing its solvent exposure. In the case of octreotide, the addition of the second label led to the formation of the dual labelled conjugate together with a side-product, assumed to be the product of a competing lysine modification. Attempts to stop the formation of the side-product were unsuccessful.



Scheme 118. Dual labelling of somatostatin

Regarding the activity of the protein after dual labelling, it is assumed that that might be severely affected as the disulphide bond remains broken. Accordingly, the strategy might be most suitable for artificially inserted disulphides or for disulphides that are not crucial for maintaining the biomolecule's activity.

A series of novel disubstituted maleimides was also synthesised and investigated as potential disulphide bridging reagents using somatostatin **29** as model system (Fig. 53). Diphenoxymaleimides are significantly slower compared to dibromo- or dithiophenolmaleimide but the reactions can be accelerated by adding excess reagent. This class of reagents is the first to display no cross-reactivity with

the reducing agent TCEP, therefore are ideal for the *in situ* protocol. Sulphoxythiomaleimides afforded the bridged somatostatin conjugate when used in excess (6 eq.) but suffered from the same aqueous instability as the sulfoxymaleimides. Halophenoxymaleimides on the other hand proved to be highly reactive, affording full conversion to the maleimide bridged peptide in just 1 min. Competition experiments with dibromomaleimide indicated that these reagents might be the fastest so far for disulphide bridging. Initial tests indicate less satisfactory conversions during the *in situ* protocol, which might suggest a certain degree of cross-reactivity between halophenoxymaleimides and TCEP but further investigation is required.

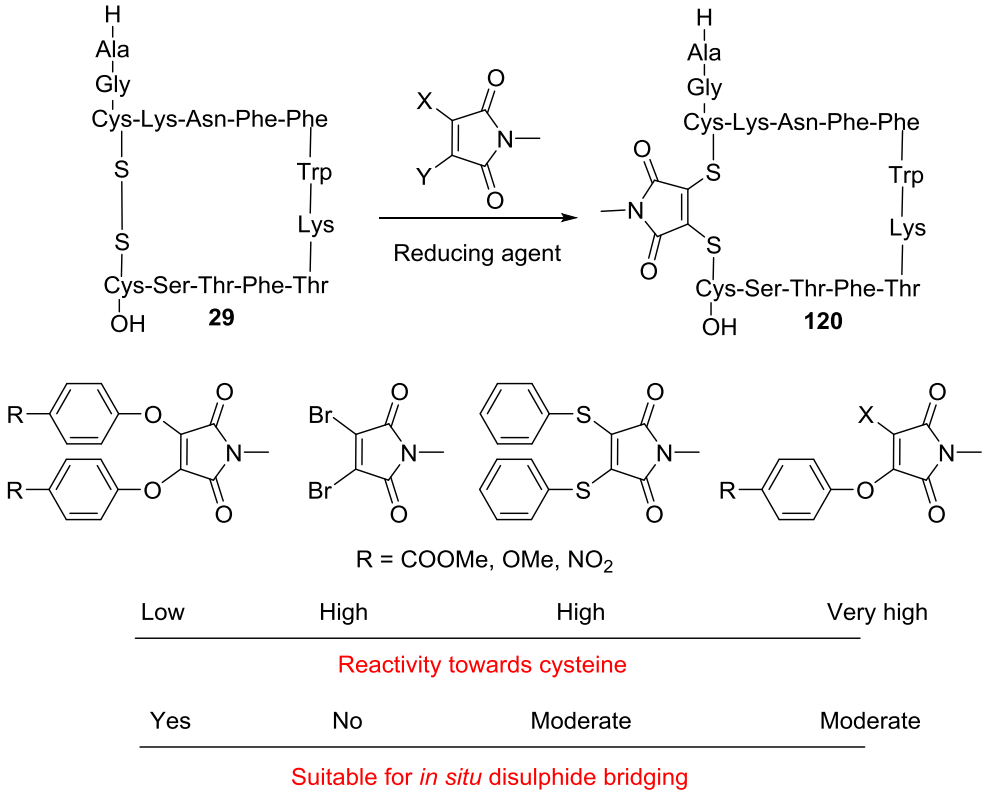
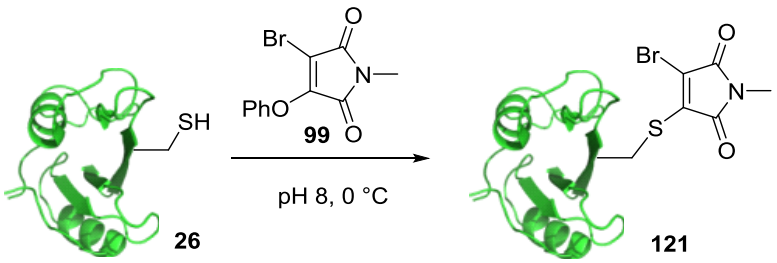


Fig. 53. Review of selected disubstituted maleimides as reagents for disulphide bridging

Finally, the regioselectivity of the thiol addition to the substituted maleimides was briefly explored. The product analysis of the reactions between the cysteine in Grb2 SH2 (L111C) **26** and substrates such as the halophenoxymaleimides and bromochloromaleimide **91** strongly suggests that the position of attack is governed by the electronic properties of the substituent (Scheme 119). Electron donating substituents will activate the α position towards nucleophilic attack *via* a mesomeric effect while electron withdrawing substituents will activate the β-position *via* the same mechanism.



Scheme 119. Exclusive α-thiol attack with respect to the oxygen in the phenoxy group

IV Future plans

Future work on this project could consist of further investigation of the cross-reactivity of halophenoxymaleimides with TCEP to establish if these reagents are compatible with the *in situ* protocol. Potentially their high reactivity could be useful in cases when the disulphide bridging is particularly difficult (e.g. when the disulphide is less explosed). By trapping the two resultant thiols very quickly, these reagents have the potential of reducing the risk of disulphide scrambling. To prove this, the model systems should contain more than one disulphide bond. Such systems, like insulin (3 disulphides) and tertiapin Q (2 disulphides) are currently under investigation in our group. The work of Dr. Felix Schumacher on insulin indicates that the scrambling of the protein during disulphide bridging is not only dependant on the labelling reagent but also the reducing reagent and the protocol used (e.g. stepwise or *in situ*). Aditionally, each protocol needs to be optimized for each protein target. The selective disulphide bridging in proteins bearing more than one disulphide bond whilst retaining biological activity would indeed be a major advancement in the field of bioconjugation, as many therapeutical proteins contain more than one disulphide (e.g. nanobodies®,[219] Fab fragments[220]).

Diphenoxymaleimides, due to their lower reactivity could afford selective labelling in systems containing multiple disulphides of varied reactivity.

The efficiency of succinimide bridging with phenoxymaleimides could potentially find applications in the context of antibody-drug conjugates. Dithiosuccinimides have been shown to be less susceptible to thiol attack compared to dithiomaleimides and this could be a very useful attribute in the context of antibody-drug conjugates where the requirements for plasma stability are very stringent.

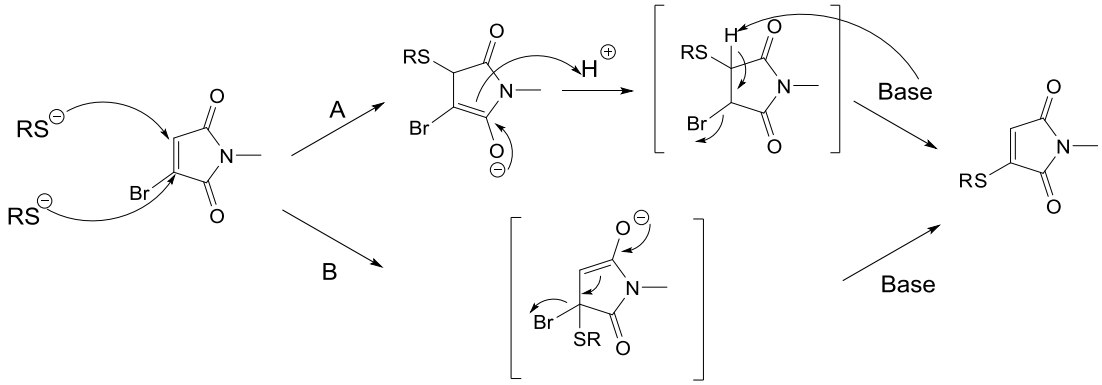
Furthermore, the retention of the biomolecule's activity after succinimide bridging should be tested, especially considering that the product is a

diastereoisomeric mixture. This aspect is paramount in the design of protein therapeutics.

Regarding the dual labelling strategy, its utility could be demonstrated on a protein substrate bearing an artificially inserted disulphide. In this way, the activity of the protein is not expected to be affected.

The competing lysine modification remains a drawback of the strategy. From the data gathered so far, it seems that the extent of lysine modification is dependant on the protein and presumably also on the position of the disulphide on the protein. The inhibition of the lysine modification might be achieved by a more thourough understanding of the influence of the disulphide bond rather than by changing the reaction conditions and the labelling reagent. This is even more important if the targeted disulphide is artificially inserted and so its position on the protein could be varied.

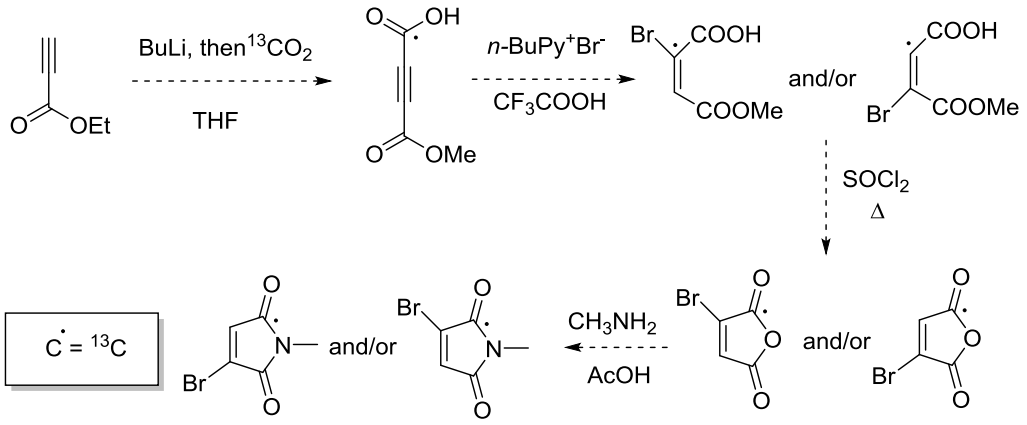
To conclude the work on the position of the thiol attack a mechanistic study is required. As seen in Scheme 120, the product of the thiol addition to bromomaleimide is the same regardless if the thiol attacks the carbon bearing the bromo substitutent (route A) or the other carbon involved in the double bond (route B).



Scheme 120. Possible mechanisms for the conjugate addition of the thiolate anion to bromomaleimide

The basis of the mechanistic study is ^{13}C-labelled bromomaleimide, for which a synthetic plan has been proposed (Scheme 121). The synthetic route starts with the carboxylation of ethyl propiolate, using ^{13}C-labelled carbon dioxide. This reaction is well documented in the literature.[221] The next step is a hydrobromination reaction, using butylpyridinium bromide.[222] The hydrobromination could lead to 4 possible products, the *Z* and *E* isomers of the two possible regioisomers. The products shown for this step are the desired products as they would allow cyclisation under standard conditions,[223] giving the bromomaleimic anhydride. The final step consists

of the condensation between methylamine and the bromomaleic anhydride, which is expected to be a high yielding reaction.[177]



Scheme 121. Proposed synthetic route for the preparation of ^{13}C-labelled bromomaleimide

V Experimental Section

V.1 Materials

All commercially available chemicals were used as received without further purification.

Lyophilised Somatostatin was purchased from Sigma Aldrich and Octreotide acetate was purchased from Source Bioscience.The anti-CEA ds-scFv shMFE23[211] antibody fragment was kindly provided by Dr Berend Tolner (UCL Cancer Institute) and the Grb 2 (L111C) SH2[139] adaptor protein was kindly provided by Dr Rachel Morgan (UCL Chemistry). Clinical grade Rituximab antibody (MabThera) was purchased from Roche. Trastuzumab (Herceptin® 150 mg, Genentech) was purchased from Roche.

All buffer solutions were prepared with double-deionised water and filter-sterilised.

V.2 General Methods

All reactions were carried out at room temperature (19 - 22 °C), under argon, unless otherwise stated.

^{1}H NMR spectra were recorded on Bruker AMX600 (600 MHz) and AMX500 (500 MHz) instruments at room temperature. The chemical shifts are expressed in parts per million (ppm) referenced to the residual solvent peaks. Data is reported as

follows: δ, chemical shift; integration, multiplicity (recorded as br (broad); s (singlet); d (doublet); t (triplet); q (quartet); qn (quintet), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of dublet of doublets), sext (sextet) and m (multiplet)), coupling constants (J in Hertz, Hz) and assignment. ^{13}C-NMR spectra were recorded on the same instruments at 150 MHz and 125 Hz respectively. The chemical shifts are expressed in parts per million (ppm), referenced to the residual solvent peaks. For NMR experiments, $CDCl_3$ denotes deuterated (d) chloroform, DMSO-d^6 denotes deuterated (d^6) dimethylsulfoxide and CD_3OD denotes deuterated (d^4) methanol.

Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR Spectrometer operating in ATR mode. Data is reported as follows: wavenumber (cm^{-1}), intensity (recorded as br (broad), s (strong), m (medium) or w (weak)). Melting points were measured with a Gallenkamp and are uncorrected. *In vacuo* is used to describe solvent evaporation by Büchi rotary evaporation between 20 - 60 °C. High and low resolution mass spectrometry was performed using a VG70 SE operating in modes ES, EI, FAB or CI depending on the sample. All reactions were monitored by thin layer chromatography (TLC), using TLC plates pre-coated with silica gel 60 F_{254} on aluminium (Merck KGaA). Chemical staining using $KMnO_4$ was employed for visualisation under UV (254 or 365 nm). Normal phase silica gel 60 (0.04-0.063 mm, 230-400 mesh) (BDH) and sand (VWR) were used for flash chromatography.

Somatostatin, octreotide, anti-CEA ds-scfv antibody fragment and their conjugates were analysed by LC-MS using a Waters Acquity UPLC connected to Waters Acquity Single Quad Detector [column: Acquity UPLC BEH C18 1.7 μm 2.1 x 50 mm; wavelength 254 nm; mobile phase 95 : 5 water (0.1% formic acid) : CH_3CN (0.1% formic acid), gradient over 4 min to 5 : 95 water (0.1 % formic acid) : CH_3CN (0.1 % formic acid); flow rate 0.6 mL/ min; MS mode ES+/-; scan range (*m/z*) = 95 - 2090 Da; scan time 0.25 s]. Data was obtained in continuum mode. Sample volumes were between 10 - 30 μL. The electron spray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 20 - 200 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Total mass spectra for protein samples were recontructed from the ion series using the MaxEnt 1 algorithm pre-installed on the MassLynx software.

Rituximab and Herceptin Fabs were analysed by LC-MS using a Thermo Scientific uPLC connected to MSQ Plus Single Quad Detector (SQD) [Column: Hypersil Gold C4, 1.9 μm 2.1 x 50 mm; wavelength: 254 nm; mobile phase: 99 : 1 water: CH_3CN (0.1% formic acid), gradient over 4 min to 1: 9 water : CH_3CN (0.1%

formic acid).; flow rate: 0.3 mL/min; MS Mode: ES+; scan range: (*m/z*) = 500 - 2000; scan time: 1.5 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 3.5 - 5.0 min range. Total mass spectra for protein samples were reconstructed from the ion series using the pre-installed ProMass software.

The retention times of protein and peptide samples is given based on the gradient detailed above.

Protein concentrations and UV-absorbances were obtained on a Carry BIO 100 UV-Vis spectrophotometer (Varian) equivuipped with a temperature controlled 12x sample holder in quartz cuvettes (1 cm path length, volume 75 μL) at room temperature, unless otherwise stated. Samples were baseline corrected.

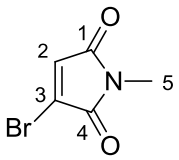
Glycine-SDS-PAGE was performed following standard lab procedures.12% gels were used, with a 4% gel as the stacking gel. A broad range MW marker (10 kDa-250 kDa, BioLabs) was used as protein ladder. Samples were mixed in 1 : 1 volume ratio with the running buffer (1 M Tris-HCl, pH 6.8, 30% w/v glycerol, 10% w/v SDS, Coomassie G-250). Coomassie G-250 was used for staining.

Reactions involving somatostatin and octreotide were performed on a 100 - 700 μL scale in 1.5 mL Eppendorf tubes while reactions involving Fab fragments and Grb2 SH2 (L111C) were performed on a 10 - 100 μL scale. Reactions with a total volume lower than 50 μL, were performed in 100 μL Eppendorf tubes.

Buffer swapping of protein solutions was performed using Vivaspin 500 (Santorius) Vivaspin 6 mL (Santorius) filters, both with 10 kDa MWCO.

V.3 Synthesised compounds

57. 3-Bromo-1-methyl-pyrrole-2,5-dione[138]

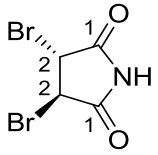


To *N*-methylmaleimide (2.87 g, 25.0 mmol) in $CHCl_3$ (125 mL), bromine (2.60 mL, 55.5 mmol) was added dropwise and the resulting mixture was refluxed for 2 h. The solvent was removed *in vacuo* and the resulting solid was dissolved in EtOAc

(30 mL) and washed with 15% aq. $Na_2S_2O_3$ (20 mL). The product was extracted with EtOAc (3 x 30 mL), dried ($MgSO_4$) and filtered. The solvent was evaporated *in vacuo* to afford 6.39 g of *N*-methyl 2,3-dibromosuccinimide as yellow crystals. The succinimide product was dissolved in AcOH (150 mL), then sodium acetate (5.94 g, 71.6 mmol) was added and the reaction mixture was refluxed for 2.5 h. The reaction mixture was left to cool down to room temperature and then the solvent was evaporated *in vacuo*. The crude mixture was dissolved in EtOAc (50 mL) and washed with sat. aq. Na_2CO_3 (3 x 25 mL). The organic phase was dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 80 : 20 to 60 : 40) afforded the title compound **57** as white crystals (2.86 g, 15.1 mmol) in 65% yield.

δ_H (600 MHz, $CDCl_3$) 6.88 (1H, s, C*H*-2), 3.07 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 168.7 (C=O), 165.5 (C=O), 132.0 (CH), 131.5 (C), 24.7 (CH_3); IR 3105 (w), 2947 (w), 1776 (m), 1704 (s), 1588 (s), 14 93 (s), 1388 (m), 1231 (m), 969 (s), 706 (s); MS (CI+) *m/z,* (relative intensity): 192 ([^{81}M+H]$^+$, 100), 190 ([^{79}M+H]$^+$, 100). Exact mass calcd. for [$C_5H_4NO_2{}^{79}$Br]$^+$+H: 189.9504. Measured: 189.9501 (CI+); m.p.: 100 °C (lit. m.p.[138]: 88 - 89 °C).

58. 3,4-dibromo-pyrrolidine-2,5-dione[138]

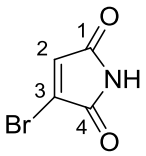


To maleimide (2.00 g, 20.0 mmol) in $CHCl_3$ (15 mL) bromine (1.16 mL, 20.0 mmol) in $CHCl_3$ (15 mL) was added dropwise. The reaction mixture was refluxed for 3 h and then allowed to cool to room temperature over 1 h. The solid yellow precipitate was filtered and washed with cold $CHCl_3$ (2 x 35 mL) to afford the title compound **58** as a white solid (3.85 g, 14.9 mmol) in 75% yield.

δ_H (500 MHz, $CDCl_3$) 8.34 (1H, br s, N*H*), 4.73 (2H, s, 2 x C*H*-2); δ_C (125 MHz, $CDCl_3$) 169.8 (2 x C=O), 42.8 (2 x CH); IR 2945 (w), 1789 (m), 1708 (s), 1373 (s), 1170 (s); MS (ES+) *m/z,* (relative intensity): 259 ([^{81,81}M+H]$^+$, 28), 257 ([^{79,81}M+H]$^+$, 29), 255 ([^{79,79}M+H]$^+$, 30); m.p.: 89 - 92 °C (lit. m.p.: 125 - 125.3 °C).

The stereochemistry of compound **58** was investigated. Based on molecular modelling (PC Model v 8.5) the calculated $^{3}J_{HH}$ for the *anti* isomer (corresponding to a torsional angle of 119°) is 2.2 Hz, exactly the same value that was measured when analysing the ^{13}C satellites in the ^{1}H-NMR spectrum. This demonstrates that the 2,3-dibromosuccinimide **58** was obtained as the *anti* isomer.

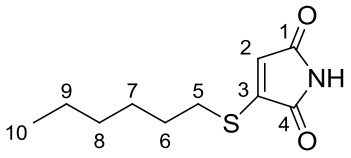
22. 3-Bromo-pyrrole-2,5-dione[138]



Dibromosuccinimide **58** (965 mg, 3.75 mmol) and sodium acetate trihydrate (1.54 g, 11.3 mmol) were dissolved in AcOH (23 mL). The reaction mixture was refluxed for 1.5 h, then allowed to cool to room temperature and then the solvent was evaporated *in vacuo*. The crude mixture was redissolved in EtOAc (50 mL) and washed with sat. aq. Na_2CO_3 (3 x 10 mL). The organic phase were dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 80 : 20 to 60 : 40) afforded the title compound **22** as white crystals (568 mg, 3.22 mmol) in 86% yield.

δ_H (500 MHz, $CDCl_3$) 7.70 (1H, br s, N*H*), 6.89 (1H, d, *J* = 1.5 Hz, C*H*-2); δ_C (150 MHz, $CDCl_3$) 168.0 (C=O), 164.9 (C=O), 132.9 (CH), 132.3 (C); IR 3236 (m), 1782 (m), 1763 (m), 1716 (s), 1577 (m), 871 (m), 718 (m); MS (EI+) *m/z,* (relative intensity): 177 ($[^{81}M]^+$, 95), 175 ($[^{79}M]^+$, 95), 134 (97), 132 (98), 106 (70), 104(70). Exact mass calcd. for $[C_4H_2NO_2{}^{79}Br]^+$: 174.92634. Measured: 174.92649 (EI+); m.p.: 149 °C (lit. m.p.[138]: 149 - 151 °C).

59. 3-Hexylthio-pyrrole-2,5-dione[177]

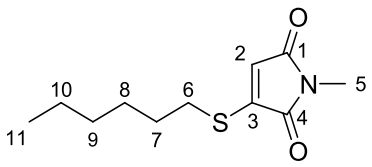


To a solution of bromomaleimide **22** (276 mg, 1.58 mmol) and sodium acetate trihydrate (215 mg, 1.58 mmol) in MeOH (45 mL) *n*-hexanethiol (250 µL, 1.72 mmol) in MeOH (300 mL) was added dropwise over 1 h with vigorous stirring. After a

further 20 min the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 20 to 70 : 30) afforded the title compound **59** as a yellow solid (337 mg, 1.58 mmol) in 85% yield.

δ_H (600 MHz, $CDCl_3$) 6.04 (1H, s, C*H*-2), 2.91 (2H, t, *J* = 7.3 Hz, CH_2-5), 1.75 (2H, qn, *J* = 7.5 Hz, CH_2-6), 1.45 (2H, qn, *J* = 7.4 Hz, CH_2-7), 1.33-1.27 (4H, m, CH_2-8 and 9), 0.90 (3H, t, *J* = 7.0 Hz, CH_3-10); δ_C (150 MHz, $CDCl_3$) 169.0 (C=O), 167.7 (C=O), 152.8 (C), 118.2 (CH), 32.1 (CH_2), 31.3 (CH_2), 28.6 (CH_2), 27.7 (CH_2), 22.6 (CH_2), 14.1 (CH_3); IR 3183 (w), 2956 (s), 2931 (m), 1742 (s), 1676 (s), 1549 (m), 1470 (m), 1390 (s), 1364 (s), 1153 (s), 770 (m); m.p.: 104 °C (lit. m.p.[177]: 99 - 101 °C).

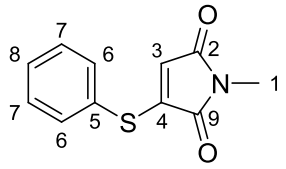
60. 3-Hexylthio-1-methyl-pyrrole-2,5-dione[177]



To a solution of **57** (300 mg, 1.58 mmol) and sodium acetate trihydrate (215 mg, 1.58 mmol) in MeOH (45 mL) *n*-hexanethiol (250 µL, 1.72 mmol) in MeOH (300 mL) was added dropwise over 1 h. After a further 15 min the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded the title compound **60** as a yellow solid (308 mg, 1.35 mmol) in 86% yield.

δ_H (600 MHz, $CDCl_3$) 6.04 (1H, s, C*H*-2), 2.90 (2H, t, *J* = 7.3 Hz, CH_2-6), 3.01 (3H, s, CH_3-5), 1.74 (2H, qn, *J* = 7.5 Hz, CH_2-7), 1.47-1.42 (2H, m, CH_2-8), 1.33-1.29 (4H, m, CH_2-9 and 10), 0.91-0.88 (3H, m, CH_3-11); δ_C (150 MHz, $CDCl_3$) 169.9 (C=O), 168.2 (C=O), 151.8 (C), 117.3 (CH), 31.9 (CH_2), 31.3 (CH_2), 28.6 (CH_2), 27.7 (CH_2), 24.1 (CH_3), 22.6 (CH_2), 14.1 (CH_3); IR 3177 (m), 2930 (m), 1796 (m), 1742 (s), 1548 (m), 1342 (m), 1033 (m), 832 (m); MS (CI+) *m/z* (relative intensity): 228 ($[M+H]^+$, 100), 194 (30). Exact mass calcd. for $[C_{11}H_{17}NO_2S]^+$+H: 228.1058. Measured: 228.1051 (CI+); m.p.: 63 °C (lit. m.p.[177]: 62 - 65 °C).

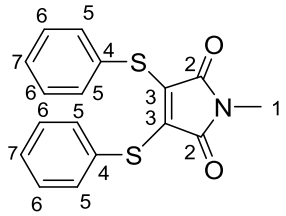
61. 1-Methyl-3-phenylthio-pyrrole-2,5-dione



To a solution of thiophenol (269 µL, 2.63 mmol) in MeOH (280 mL) was added sodium acetate (215.9 mg, 2.63 mmol). The reaction mixture was cooled to -60 °C and bromomaleimide **57** (500 mg, 2.63 mmol) in MeOH (280 mL) was added dropwise over 45 min with vigorous stirring while maintaining the temperature between -60 and -50 °C. The reaction mixture was removed from the cooling bath and was stirred for a further 10 min before the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 95 : 5 to 93 : 7) afforded the title compound **61** as a yellow oil (353 mg, 1.61 mmol) in 61% yield.

δ_H (600 MHz, $CDCl_3$) 7.55-7.54 (2H, m, 2 x C*H*-Ar), 7.49-7.47 (3H, m, 3 x C*H*-Ar), 5.65 (1H, s, C*H*-3), 3.02 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 169.7 (C=O), 168.1 (C=O), 152.7 (C), 134. 5 (2 x CH), 130.5 (CH), 130.3 (2 x CH), 127.4 (C), 118.9 (CH), 24.7 (CH_3); IR 2947 (w), 1770 (m), 1698 (s), 1561 (m), 1440 (m), 1385 (m), 971 (m); MS (EI+) *m/z* (relative intensity): 219 ($[M]^+$, 27), 134 (100), 90 (23). Exact mass calcd. for $[C_{11}H_9NO_2S]^+$: 219.0348. Measured: 219.0345 (EI+).

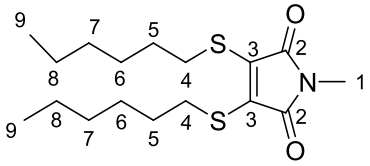
63. 1-Methyl-3,4-*bis*(phenylthio)-pyrrole-2,5-dione



A solution of thiophenol (256 µL, 3.35 mmol) in MeOH (3 mL) was added dropwise to a solution of *N*-methyl 2,3-dibromomaleimide (400 mg, 1.47 mmol) and sodium acetate (370 mg, 3.35 mmol) in MeOH (28 mL). The reaction mixture was stirred at room temperature for 15 min before the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 94 : 6 to 90 : 10) afforded title compound **63** as a yellow solid (399 mg, 1.22 mmol) in 85% yield.

δ_H (600 MHz, $CDCl_3$) 7.30-7.29 (2H, m, 2 x C*H*-Ar), 7.27-7.24 (4H, m, 4 x C*H*-Ar), 7.22-7.20 (4H, m, 4 x C*H*-Ar), 3.01 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 167.0 (2 x C=O), 135.9 (2 x C), 132.0 (4 x CH), 129.1 (4 x CH), 128.5 (2 x CH and 2 x C), 24.8 (CH_3); IR 1706 (s), 1440 (m), 1382 (m); MS (EI+) *m/z*, (relative intensity): 327 ($[M]^+$, 23), 180 (13), 111 (53), 107 (34); Exact mass calcd. for $[C_{17}H_{13}NO_2S_2]^+$: 327.0382. Measured: 327.0384 (EI+); m.p.: 94 - 95 °C.

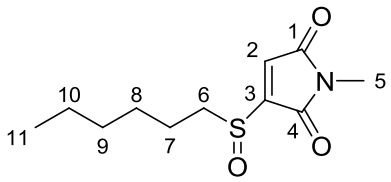
64. 3,4-*bis*(Hexylthio)-1-methyl-pyrrole-2,5-dione



A solution of *n*-hexanethiol (491 µL, 3.35 mmol) in MeOH (5 mL) was added slowly to a solution of *N*-methyl 2,3-dibromomaleimide (400 mg, 1.47 mmol) and sodium acetate (275 mg, 3.35 mmol) in MeOH (29 mL). The reaction mixture was stirred for 15 min at room temperature and then the solvent was evaporated *in vacuo.* Purification by flash chromatography (PE : Et_2O, 99 : 1) afforded title compound **64** as a yellow oil (170 mg, 0.49 mmol) in 34% yield.

δ_H (600 MHz, $CDCl_3$) 3.26 (4H, t, *J* = 7.5 Hz, 2 x CH_2-4), 3.00 (3H, s, CH_3-1), 1.63 (4H, qn, *J* = 7.5 Hz, 2 x CH_2-5), 1.41 (4H, qn, *J* = 7.4, Hz, 2 x CH_2-6), 1.31-1.25 (8H, m, 2 x CH_2-7 and 2 x CH_2-8), 0.88 (6H, t, *J* = 7.0 Hz, 2 x CH_3-9); δ_C (150 MHz, $CDCl_3$) 166.9 (2 x C=O), 136.0 (2 x C), 32.0 (2 x CH_2), 31.4 (2 x CH_2), 30.6 (2 x CH_2), 28.3 (2 x CH_2), 24.5 (CH_3), 22.6 (2 x CH_2), 14.1 (2 x CH_3); IR 2925 (w), 1721 (s), 1637 (s), 1439 (m), 1148 (m); MS (EI+) *m/z* (relative intensity): 343 ($[M]^+$, 100), 226 (26), 175 (22). Exact mass calcd. for $[C_{17}H_{29}NO_2S_2]^+$: 343.1634. Measured: 343.1638 (EI+).

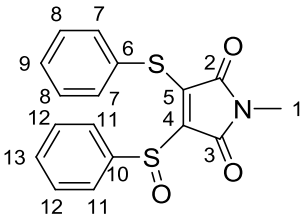
66. 3-Hexylsulfinyl-1-methyl-pyrrole-2,5-dione



To a solution of thiomaleimide **60** (300 mg, 1.32 mmol) in CH_2Cl_2 (30 ml) at -10 °C a solution of *m*-CPBA (235 mg, 1.32 mmol) in CH_2Cl_2 (15 ml) was added over 30 min. A further portion of *m*-CPBA (70.4 mg, 0.39 mmol) in CH_2Cl_2 (3 mL) was added after 3 h. Another portion of *m*-CPBA (47.0 mg, 0.26 mmol) in CH_2Cl_2 (1 mL) was added after 5 h. After a total of 6 h the reaction mixture was washed with 10% aq. $NaHCO_3$ (2 x 25 mL), dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo* to afford the title compound **66** as a pale yellow waxy solid (321 mg, 1.32 mmol) in 100% yield.

δ_H (600 MHz, $CDCl_3$) 7.13 (1H, s, C*H*-2), 3.20 (1H, ddd, *J* = 7.3, 6.0, 4.3 Hz, C*H*H-6), 3.06 (3H, s, CH_3-5), 3.05-3.01 (1H, m, CH*H*-6), 1.99-1.91 (1H, m, CH*H*-7), 1.73-1.66 (1H, m, C*H*H-7), 1.51-1.43 (2H, m, CH_2), 1.34-1.30 (4H, m, 2 x CH_2), 0.91-0.88 (3H, m, CH_3-11); δ_C (150 MHz, $CDCl_3$) 167.0 (C=O), 166.5 (C=O), 154.7 (C), 133.8 (CH), 53.4 (CH_2), 31.2 (CH_2), 28.3 (CH_2), 24.4 (CH_3), 22.5 (CH_2), 22.0 (CH_2), 14.1 (CH_3); IR 1708 (s), 1442 (m), 1383 (m); MS (CI+) *m/z* (relative intensity): 244 ($[M+H]^+$, 100), 117 (12). Exact mass calcd. for $[C_{11}H_{17}NO_3S]^+$+H: 244.1001. Measured: 244.1002 (CI+); m.p.: 33 - 35 °C.

68. 1-Methyl-3-phenylsulfinyl-4-phenylthio-pyrrole-2,5-dione

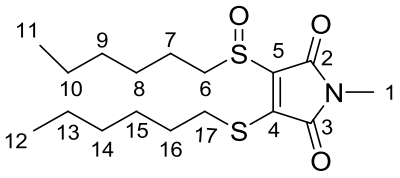


To a solution of dithiomaleimide **63** (100 mg, 0.30 mmol) in CH_2Cl_2 (7 mL) was added *m*-CPBA (105 mg, 0.61 mmol) in CH_2Cl_2 (3.5 mL) over 30 min at -10°C. The reaction mixture was allowed to warm to room temperature over 1.5 h. After this time, the solution was washed with 10% aq. Na_2CO_3 (5 mL), brine (5 mL), dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded the title compound **68** as a yellow oil (48.7 mg, 0.14 mmol) in a 46% yield.

δ_H (600 MHz, $CDCl_3$) 7.68-7.65 (4H, m, 4 x C*H*-Ar), 7.53-7.49 (4H, m, 4 x C*H*-Ar), 7.48-7.45 (2H, m, 2 x C*H*-Ar), 2.88 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 167.7 (C=O), 167.6 (C=O), 150.0 (C), 141.6 (C), 134.8 (2 x CH-Ar), 133.2 (C), 131.5 (CH),

131.3 (CH), 130.3 (2 x CH), 129.5 (2 x CH), 126.6 (C), 124.8 (2 x CH), 24.6 (CH_3); IR 1775 (w), 1707 (s), 1532 (w), 1442 (m), 1380 (m); MS (CI+) *m/z* (relative intensity): 344 ($[M+H]^+$, 100), 327 (14), 295 (27). Exact mass calcd. for $[C_{17}H_{13}NO_3S_2]^+$+H: 344.0415. Measured: 344.0419 (CI+).

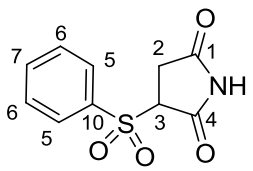
69. 3-Hexylsulfinyl-4-hexylthio-1-methyl-pyrrole-2,5-dione



To a solution of dithiomaleimide **64** (128 mg, 0.37 mmol) in CH_2Cl_2 (9 mL) was added *m*-CPBA (146 mg, 0.82 mmol) in CH_2Cl_2 (5 mL) over 30 min at -15°C. The reaction mixture was allowed to warm to room temperature over 45 min. After this time, the mixture was washed with 10% aq. Na_2CO_3 (5 mL), brine (5 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 85 : 15 to 70 : 30) afforded the title compound **69** as a yellow oil (50.7 mg, 0.14 mmol) in 38% yield.

δ_H (600 MHz, $CDCl_3$) 3.44 (2H, td, *J* = 11.5 , 3.5 Hz, C*H*$_2$-17), 3.39-3.34 (1H, ddd, *J* = 15.2, 9.6, 5.6 Hz, CH*H*-6), 3.18-3.13 (1H, ddd, *J* = 16.2, 9.7, 6.4 Hz, C*H*H-6), 3.03 (3H, s, C*H*$_3$-1), 1.82-1.73 (2H, m, C*H*$_2$-7), 1.70 (2H, qn, *J* = 7.7 Hz, C*H*$_2$-16), 1.49-1.41 (4H, m, C*H*$_2$-8 and C*H*$_2$-15), 1.33-1.28 (8H, m, C*H*$_2$-9, C*H*$_2$-10, C*H*$_2$-13, C*H*$_2$-14), 0.89 (6H, t, *J* = 6.9 Hz, C*H*$_3$-11 and C*H*$_3$-12); δ_C (150 MHz, $CDCl_3$) 165.4 (C=O), 165.1 (C=O), 151.6 (C), 130.5 (C), 52.6 (CH_2), 32.3 (CH_2), 31.5 (CH_2), 31.3 (CH_2), 29.9 (CH_2), 28.46 (CH_2), 28.43 (CH_2), 24.6 (CH_3), 22.8 (CH_2), 22.6 (CH_2), 22.5 (CH_2), 14.1 (CH_3), 14.0 (CH_3); IR 2952 (m), 2926 (m), 1704 (s), 1526 (w), 1434 (m), 1379 (m), 1051 (m); MS (EI+) *m/z* (relative intensity): 359 ($[M]^+$, 15), 342 (46) 275 (48), 175 (100), 116 (78). Exact mass calcd. for $[C_{17}H_{19}NO_3S_2]^+$: 359.1583. Measured: 359.1577 (EI+).

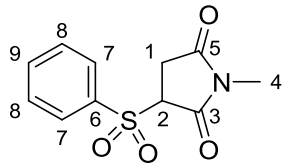
70. 3-Phenylsulfonyl-pyrrolidine-2,5-dione[180]



To a solution of maleimide (500 mg, 5.15 mmol) in water (10 mL) monopotassium phosphate buffer (10% (w/v) aq., 84 mL, 6.18 mmol) and sodium benzenesulfinate (950 mg, 5.67 mmol) were added. The resulting mixture was stirred at room temperature for 2 h, during which time a white solid precipitated. The solid was filtered and dried *in vacuo* to give the title compound **70** as a white solid (1.20 g, 5.01 mmol) in 98% yield.

δ_H (600 MHz, $CDCl_3$) 8.03 (1H, br s, N*H*), 7.96-7.94 (2H, m, 2 x C*H*-5), 7.78-7.75 (1H, m, C*H*-7), 7.65-7.62 (2H, m, 2 x C*H*-6), 4.36 (1H, dd, *J* = 9.7, 3.9 Hz, C*H*-3), 3.41 (1H, dd, *J* = 19.2, 3.8 Hz, C*H*H-2), 3.12 (1H, dd, *J* = 19.2, 9.8 Hz, CH*H*-2); δ_C (150 MHz, $CDCl_3$) 172.3 (C=O), 167.9 (C=O), 136.4 (C), 135.2 (CH), 129.6 (2 x CH), 129.5 (2 x CH), 64.5 (CH), 31.0 (CH_2); IR 3342 (w), 1792 (m), 1714 (s), 1311 (m), 1150 (m); MS (EI+) *m/z* (relative intensity): 239 ($[M]^+$, 4), 175 (55), 141 (85), 85 (100). Exact mass calcd. for $[C_{10}H_9NO_4S]^+$: 239.0247. Measured: 239.0248 (EI+); m.p.: 152 °C (lit. m.p.[180]: 156 - 158 °C).

71. 1-Methyl-3-phenylsulfonyl-pyrrolidine-2,5-dione

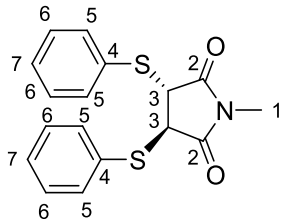


To a solution of *N*-methylmaleimide (111 mg, 1.00 mmol) in water (1.8 mL) monopotassium phosphate buffer (10% (w/v) aqueous, 1.5 mL, 1.10 mmol) and sodium benzenesulfinate (201 mg, 1.20 mmol) were added. The mixture was stirred at room temperature for 1.5 h. Firstly a white solid precipitated, but in time the reaction mixture turned pink and a waxy solid was formed. The reaction mixture was extracted with CH_2Cl_2 (3 x 10 mL), the combined organic layers were washed with brine (5 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo* to afford the title compound **71** as a white solid (228 mg, 0.90 mmol) in a 90% yield.

δ_H (600 Hz, $CDCl_3$) 7.94-7.93 (2H, m, 2 x C*H*-7), 7.75 (1H, tt, *J* = 7.5, 1.3 Hz, C*H*-9), 7.65-7.62 (2H, m, 2 x C*H*-8), 4.33 (1H, dd, *J* = 9.6, 3.8 Hz, C*H*-2), 3.36 (1H, dd, *J* = 19.0, 3.8 Hz, C*H*H-1), 3.07 (1H, dd, *J* = 19.0, 9.6 Hz, CH*H*-1), 2.97 (3H, s, CH_3-4); δ_C (150 Hz, $CDCl_3$) 172.9 (C=O), 168.6 (C=O), 136.6 (C), 135.1 (CH), 129.6 (2 x CH), 129.5 (2 x CH), 63.4 (CH), 31.0 (CH_2), 25.6 (CH_3); IR 2950 (w), 1784 (w), 1702

(s), 1436 (m), 1382 (m), 1323 (m), 1311 (m), 1286 (m), 1150 (s), 1117 (m); MS (EI+) *m/z* (relative intensity): 252 ($[M]^+$, 100). Exact mass calcd. for $[C_{11}H_{11}NO_4S]^+$: 252.0331. Measured: 252.0334 (EI+); m.p.: 147 - 149 °C.

62. (3r,4r)-1-Methyl-3,4-bis(phenylthio)pyrrolidine-2,5-dione



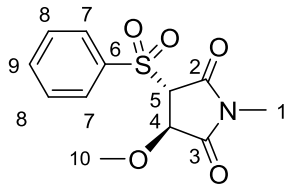
A solution of thiophenol (269 µL, 2.63 mmol) in MeOH (100 mL) was added over 15 min to a solution of bromomaleimide **57** (250 mg, 1.31 mmol) and sodium acetate (179 mg, 1.31 mmol) in MeOH (70 mL). The reaction mixture was stirred at room temperature for 18 h and then the solvent was removed *in vacuo*. Water (10 mL) was added and then the product was extracted with EtOAc (3 x 10 mL), washed with brine (10 mL), dried ($MgSO_4$) and filtered. The solvent was evaporated in vacuo to afford the title compound **62** as a yellow solid (410 mg, 1.24 mmol) in 95% yield.

δ_H (600 MHz, $CDCl_3$) 7.45-7.44 (4H, m, 4 x CH-Ar), 7.38-7.35 (2H, m, 2 x CH-5), 7.33-7.30 (4H, m, 4 x CH-Ar), 3.90 (2H, s, 2 x CH-3), 2.74 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$)* 173.4 (2 x C=O), 134.9 (4 x CH), 129.8 (2 x CH), 129.6 (4 x CH), 51.8 (2 x CH), 25.4 (CH_3); IR 2970 (w), 1737 (s), 1714 (s), 1438 (m), 1365 (m), 1228 (m), 1217 (m), 745 (w); MS (EI+) m/z, (relative intensity): 329 ($[M]^+$, 3), 220 (14), 109 (100). Exact mass calcd. for $[C_{17}H_{15}NO_2S_2]^+$: 329.05387. Measured: 329.05426 (EI+); m.p.: 94 - 96 °C.

* 2 C missing due to overlap

The stereochemistry of compound x was investigated. Based on molecular modelling (PC Model v 8.5) $^3J_{HH}$ for the anti isomer (corresponding to a torsional angle of 118°) is 3.2 Hz, whereas for the syn isomer $^3J_{HH}$ (corresponding to a torsional angle of 5°) is 11.1 Hz. From the ^{13}C satellites in the ^1H-NMR spectrum, $^3J_{HH}$ = 3.2 Hz which demonstrates that compound **62** was obtained as the *anti* isomer.

76. 3-Methoxy-1-methyl-4-phenylsulfonyl-pyrrolidine-2,5-dione

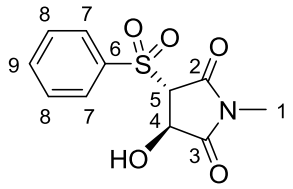


To a solution of dithiosuccinimide **62** (20.0 mg, 0.06 mmol) in MeOH (7.5 mL) was added Oxone® (221.3 mg, 0.36 mmol) in water (2.5 mL) and the resulting mixture was stirred at room temperature for 12 h. After this time, the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 70 : 30 to 50 : 50) afforded the title compound **76** as a transparent oil (3.80 mg, 0.01 mmol) in 22% yield.

δ_H (600 MHz, $CDCl_3$) 7.98-7.97 (2H, m, 2 x C*H*-Ar), 7.77-7.75 (1H, m, C*H*-Ar), 7.66-7.62 (2H, m, 2 x C*H*-Ar), 4.71 (1H, d, *J* = 3.5 Hz, C*H*-4 or C*H*-5), 4.17 (1H, d, *J* = 3.5 Hz, C*H*-4 or C*H*-5), 3.69 (3H, s, CH_3-10), 3.00 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 172.2 (C=O), 166.5 (C=O),137.1 (C), 135.2 (CH), 129.5 (2 x CH), 129.4 (2 x CH), 75.4 (CH), 69.9 (CH), 60.4 (CH_3), 24.2 (CH_3); IR 2923 (w), 1710 (s), 1434 (m), 13412 (m), 1151 (s); MS (CI+) *m/z* (relative intensity): 284 ($[M+H]^+$, 100), 251 (20). Exact mass calcd. for $[C_{12}H_{13}NO_5S]^+$+H: 284.0593. Measured: 284.0588 (CI+).

From molecular modelling of compound **76**; the deheadral angle is 130 °C in the *trans* isomer and 20 °C in the *cis* isomer. These two angles correspond to 4.5 Hz and 10 Hz respectively. The J coupling is 3.5 Hz for the dublet in compound **76**, indicating the *trans* isomer.

78. 3-hydroxy-1-methyl-4-phenylsulfonyl-pyrrolidine-2,5-dione

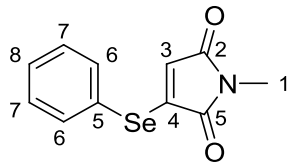


To a solution of dithiosuccinimide **62** (20.0 mg, 0.06 mmol) in *t*-BuOH (7.5 mL) was added Oxone® (221 mg, 0.36 mmol) in water (2.5 mL) and the resulting mixture was stirred at room temperature for 12 h. After this time, the solvent was evaporated *in*

vacuo. Purification by flash chromatography (PE : EtOAc, gradient elution from 70 : 30 to 50 : 50) afforded the title compound **78** as a transparent oil (4.80 mg, 0.02 mmol) in 30% yield.

δ_H (600 MHz, $CDCl_3$) 8.00-7.99 (2H, m, 2 x C*H*-Ar), 7.80-7.78 (1H, m, C*H*-Ar), 7.69-7.67 (2H, m, 2 x C*H*-Ar), 4.94 (1H, d, *J* = 3.5 Hz, C*H*-4), 2.98 (3H, s, CH_3-1); (150 MHz, $CDCl_3$) 175.9 (C=O), 168.2 (C=O),139.2 (C), 135.9 (CH), 130.5 (2 x CH), 130.4 (2 x CH), 71.6 (CD), 68.9 (CH), 60.3 (CH_3), 25.4 (CH_3); IR 1713 (s), 1448 (m), 1311 (m), 1152 (s); MS (CI+) *m/z* (relative intensity): 270 ([M+H]$^+$, 50), 205 (100). Exact mass calcd. for $[C_{11}H_{11}NSO_5]^+$+H: 270.0436. Measured: 270.0438 (CI+).

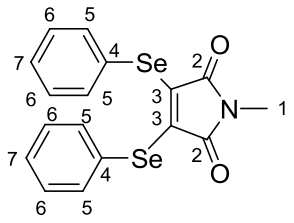
79. 1-Methyl-3-phenylselanyl-pyrrole-2,5-dione



To a degassed solution of benzeneselenol (604 µL, 2.52 mmol) in MeOH (220 mL) was added sodium acetate (172 mg, 2.10 mmol) and the reaction mixture was cooled to -60°C. A solution of bromomaleimide **57** (200 mg, 1.05 mmol) in MeOH (220 mL) was added dropwise over 45 min with vigorous stirring while maintaining the temperature between -60 and -50 °C. The reaction mixture was allowed to warm to room temperature over 15 min and then the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded of the title compound **79** as a yellow oil (514 mg, 1.93 mmol) in 92% yield.

δ_H (600 MHz, $CDCl_3$) 7.63-7.62 (2H, m, 2 x C*H*-Ar), 7.49-7.46 (1H, m, C*H*-8), 7.22-7.20 (2H, m, 2 x C*H*-Ar), 5.88 (1H, s, C*H*-3), 3.02 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 169.9 (C=O), 169.2 (C=O), 150.6 (C), 135.9 (2 x CH), 130.4 (2 x CH), 130.2 (CH), 124.7 (CH), 123.9 (C), 24.2 (CH_3); IR 1765 (w), 1701 (s), 1556 (w), 1440 (m), 1384 (m); MS (EI+) *m/z* (relative intensity): 267 ([M]$^+$, 84), 209 (32), 182 (83), 102 (100). Exact mass calcd. for $[C_{11}H_9NO_2Se]^+$: 266.9793. Measured: 266.9802 (EI+).

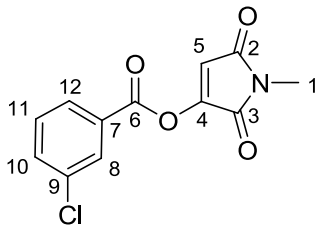
80. 1-Methyl-3,4-*bis*(phenylselanyl)-pyrrole-2,5-dione



A degassed solution of benzeneselenol (425 µL, 3.89 mmol) in MeOH (3 mL) was added dropwise to a solution of *N*-methyl 2,3-dibromomaleimide (400 mg, 1.47 mmol) and sodium acetate (320 mg, 3.90 mmol) in MeOH (47 mL). After 1 h, a second portion of sodium acetate (121 mg, 1.47 mmol) and benzeneselenol (100 µL, 0.91 mmol) in MeOH (0.5 mL) was added. After 2 h, a third portion of benzeneselenol (161 µL, 1.47 mmol) in MeOH (0.5 mL) was added and the reaction mixture was stirred for a further 10 min before the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 95 : 5 to 92 : 8) afforded the title compound **80** as a yellow solid (369 mg, 0.87 mmol) in 61% yield.

δ_H (600 MHz, $CDCl_3$) 7.49-7.47 (4H, m, 4 x C*H*-Ar), 7.36-7.33 (2H, m, 2 x C*H*-7), 7.29-7.27 (4H, m, 4 x C*H*-Ar), 2.97 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 167.6 (2 x C=O), 138.5 (2 x C), 134.9 (4 x CH), 129.4 (4 x CH), 128.9 (2 x CH), 126.2 (2 x C), 24.8 (CH_3); IR 1717 (s), 1578 (w), 1439 (m), 1473 (w), 439 (m), 2243 (s); MS (EI+) *m/z* (relative intensity): 423 ([M]$^+$, 25), 314 (16), 111 (32). Exact mass calcd. for $[C_{17}H_{13}NO_2Se_2]^+$: 422.9271. Measured: 422.9278 (EI+); m.p.: 82 - 83 °C.

82. 1-Methyl-pyrrole-2,5-dione-3-yl 3-chlorobenzoate

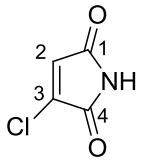


To a solution of seleomaleimide **79** (64.8 mg, 0.24 mmol) in CH_2Cl_2 (5 mL) was added *m*-CPBA (43.3 mg, 0.24 mmol) in CH_2Cl_2 (3 mL) over 30 min at -15 °C. The reaction mixture was allowed to warm to room temperature and after 3 h a second portion of *m*-CPBA (21.6 mg, 0.12 mmol) in CH_2Cl_2 (1.0 mL) was added dropwise at

-15 °C. After a further 20 h a third portion of *m*-CPBA (13.0 mg, 0.07 mmol) in CH_2Cl_2 (0.5 mL) was added dropwise at -15 °C. After a total of 22 h, the reaction mixture was washed with 10% aq. Na_2CO_3 (5 mL), brine (5 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded the title compound **82** as a white solid (7.40 mg, 0.03 mmol) in 11% yield.

δ_H (600 MHz, $CDCl_3$) 8.16 (1H, t, *J* = 1.7 Hz, C*H*-8), 8.09 (1H, ddd, *J* = 7.8, 1.2, 1.1 Hz, C*H*-12), 7.67 (1H, ddd, *J* = 8.0, 1.1, 0.9 Hz, C*H*-10), 7.49 (1H, t, *J* = 7.9 Hz, C*H*-11), 6.67 (1H, s, C*H*-5), 3.08 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 169.9 (C=O), 165.6 (C=O), 161.1 (C=O), 149.6 (C), 135.4 (C), 135.1 (CH), 130.7 (CH), 130.4 (CH), 129.0 (C), 128.9 (CH), 111.2 (CH), 24.1 (CH_3); IR 3167 (m), 1750 (s), 1711 (s), 1629 (m); MS (CI+) *m/z,* (relative intensity): 266 ($[M+H]^+$, 41), 139 (100). Exact mass calcd. for $[C_{12}H_8ClNO_4]^+$+H: 266.0220. Measured: 266.0219 (CI+); m.p. 123 - 125 °C.

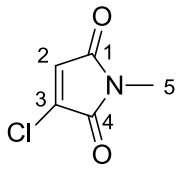
85. 3-Chloro-pyrrole-2,5-dione[224]



To a solution of bromomaleimide **22** (75.0 mg, 0.43 mmol) in MeOH (1 mL) was added tetrabutylammonium chloride (953 mg, 3.43 mmol) and the reaction mixture was refluxed. After 12 h, the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 80 : 20 to 70 : 30) afforded the title compound **85** as a white solid (33.0 mg, 0.25 mmol) in 58% yield.

δ_H (500 MHz, $CDCl_3$) 7.46 (1H, br s, N*H*), 6.66 (1H, s, C*H*-2); δ_C (125 MHz, $CDCl_3$) 166.9 (C=O), 164.4 (C=O), 141.7 (C), 127.9 (CH); IR 3237 (m), 1720 (s), 1590 (m), 1036 (w); MS (CI+) *m/z*, (relative intensity): 134 ($[^{37}M+H]^+$, 30), 132 ($[^{35}M+H]^+$, 100). Exact mass calcd. for $[C_4H_3NO_2{}^{35}Cl]^+$+H: 131.9852. Measured: 131.9855 (CI+); m.p.: 130 °C (lit. m.p.[225]: 131 °C).

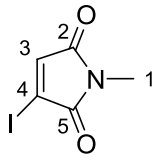
86. 3-Chloro-1-methyl-pyrrole-2,5-dione



To a solution of bromomaleimide **57** (400 mg, 2.10 mmol) in MeOH (4 mL) tetrabutylammonium chloride (1.17 g, 4.20 mmol) was added and the reaction mixture was refluxed. After 4.5 h a second portion of tetrabutylammonium chloride (2.28 g, 8.20 mmol) was added and the reflux was continued. After a total of 7.5 h, the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 95 : 5 to 75 : 25) afforded the title compound **86** as a white solid (272 mg, 1.86 mmol) in a 89% yield.

δ_H (600 MHz, $CDCl_3$) 6.64 (1H, s, C*H*-2), 3.07 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 168.0 (C=O), 165.1 (C=O), 141.1 (C), 126.9 (CH); 24.6 (CH_3); IR 3099 (w), 2924 (br), 1704 (s), 1601 (m), 1442 (m), 977 (m); MS (EI+) *m/z*, (relative intensity): 147 ($[^{37}M]^+$, 30), 145 ($[^{35}M]^+$, 100),. Exact mass calcd. for $[C_5H_4NO_2{}^{35}Cl]^+$: 144.9925. Measured: 144.9928 (EI+); m.p.: 115 °C.

88. 3-Iodo-1-methyl-pyrrole-2,5-dione

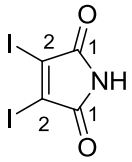


A solution of sodium iodide (2.84 g, 18.9 mmol) and bromomaleimide **57**(0.60 g, 3.15 mmol) in acetone (3 mL) was refluxed for 7 h and then stirred at room temperature for 15 h. The solids were filtered and the filtrate was concentrated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 80 : 20) afforded the title compound **88** as a yellow solid (616 mg, 2.59 mmol) in 82% yield.

δ_H (500 MHz, $CDCl_3$) 7.18 (1H, s, C*H*-3), 3.09 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 170.0 (C=O), 166.9 (C=O), 140.7 (CH), 107.3 (C), 24.9 (CH_3); IR 2922 (w), 1702 (s);

MS (EI+) *m/z* (relative intensity): 237 ([M]$^+$, 100), 152 (31); Exact mass calcd. for [$C_5H_4NO_2I$]$^+$: 236.9281. Measured: 236.9285; m.p.: 109 - 110 °C.

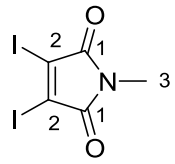
89. 3,4-Diiodo-pyrrole-2,5-dione[189]



A solution of 2,3-dibromomaleimide (1.00 g, 3.92 mmol) and sodium iodide (1.76 g, 11.8 mmol) in AcOH (14 mL) was refluxed for 2 h and then water (11 mL) was added. The white precipitate was filtered, washed with water (10 mL) and dried *in vacuo* to afford the title compound **89** as a yellow solid (1.01 g, 2.90 mmol) in 74% yield.

δ_C (150 MHz, $CDCl_3$) 165.9 (2 x C=O), 118.5 (2 x C); IR 3242 (s), 1756 (m), 1709 (s), 1545 (m), 1009 (s); MS (EI+) *m/z*, (relative intensity): 349 ([M]$^+$, 100), 179 (27) . Exact mass calcd. for [$C_4HNO_2I_2$]$^+$: 348.8091; Measured: 348.8096 (EI+); m.p.: 245 - 246 °C (lit. m.p.[189]: 254 - 255 °C).

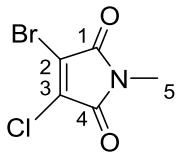
90. 3,4-Diiodo-1-methyl-pyrrole-2,5-dione



A solution of *N*-methyl 2,3-dibromomaleimide (1.00 g, 3.72 mmol) and sodium iodide (1.67 g, 11.2 mmol) in AcOH (14 mL) was refluxed for 2 h and then water (11 mL) was added. The white precipitate was filtered, washed with water (20 mL) and dried *in vacuo* to afford the title compound **90** as a yellow solid (941 mg, 2.59 mmol) in 70% yield.

δ_H (600 MHz, $CDCl_3$) 3.15 (3H, s, C*H*$_3$-3); δ_C (150 MHz, $CDCl_3$) 166.6 (2 x C=O), 117.3 (2 x C), 26.1 (CH_3); IR 1701 (s), 1553 (w), 1382 (m); MS (EI+) *m/z*, (relative intensity): 363 ([M]$^+$, 8), 127 (100). Exact mass calcd. for [$C_5H_3NO_2I_2$]$^+$: 362.8248; Measured: 362.8254 (EI+); m.p.: 250 °C.

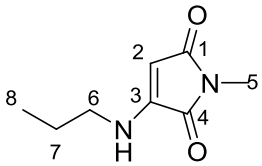
91. 3-Bromo-4-chloro-1-methyl-pyrrole-2,5-dione



To a solution of chloromaleimide **86** (75 mg, 0.51 mmol) in $CHCl_3$ (0.4 mL) a solution of bromine (26.2 µL, 0.51 mmol) in $CHCl_3$ (0.4 mL) was added dropwise and the resulting reaction mixture was refluxed for 2 h. After this time, another portion of bromine (13.1 µL, 0.5 mmol) in $CHCl_3$ (0.5 mL) was added and the reflux continued for 1 h. Then, another portion of bromine (26.2 µL, 0.51 mmol) in $CHCl_3$ (0.5 mL) was added and the reflux continued. After a total of 5 h, the solvent was removed *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 80 : 20) afforded the title compound **91** as a white solid (36.0 mg, 0.12 mmol) in 23% yield.

δ_H (600 MHz, $CDCl_3$) 3.12 (3H, s, C*H*$_3$-5); δ_C (150 MHz, $CDCl_3$) 163.64 (C=O), 164.59 (C=O), 138.2 (C), 124.4 (C), 25.4 (CH_3); IR 1719 (s), 1002 (m); MS (CI+) *m/z,* (relative intensity): 228 (25), 226 (100), 224 (76). Exact mass calcd. for $[C_5H_3{}^{79}Br{}^{35}ClNO_2]^+$: 222.9030. Measured: 222.9037 (EI+); m.p.: 130 °C.

92. 1-Methyl-3-propylamino-pyrrole-2,5-dione[177]

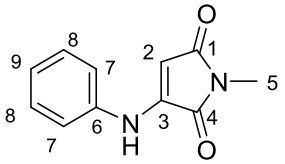


To a solution of propylamine (75 µL, 1.09 mmol) and sodium acetate (92 mg, 1.12 mmol) in MeOH (15 mL) bromomaleimide **92** (213 mg, 1.12 mmol) in MeOH (15 mL) was added dropwise and the solution was stirred at room temperature for 25 min. After this time, the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 80 : 20 to 60 : 40) afforded the title compound **92** as a yellow solid (75.1 mg, 0.45 mmol) in 41% yield.

δ_H (500 MHz, $CDCl_3$) 5.37 (1H, br s, N*H*), 4.79 (1H, s, C*H*-2), 3.15-3.11 (2H, dt, *J* = 6.9, 6.0 Hz, C*H*$_2$-6), 2.97 (3H, s, C*H*$_3$-5), 1.66 (2H, sext., *J* = 7.2 Hz, C*H*$_2$-7), 0.98

(3H, t, *J* = 7.3 Hz, C*H*$_3$-8); δ_C (150 MHz, $CDCl_3$) 172.7 (C=O), 167.7 (C=O), 149.5 (C), 83.9 (CH), 46.1 (CH_2), 23.5 (CH_3), 21.9 (CH_2), 11.4 (CH_3); IR 3317 (s), 1697 (s), 1654 (s), 1453 (m); MS (EI+) *m/z*, (relative intensity): 168 ([M]$^+$, 78), 139 (100). Exact mass calcd. for $[C_8H_{12}N_2O_2]^+$: 168.0893. Measured: 168.0889 (EI+); m.p.: 85 - 86 °C.

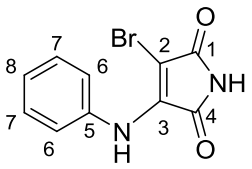
93. 1-Methyl-3-phenylamino-pyrrole-2,5-dione



To bromomaleimide **57** (100 mg, 0.53 mmol) in MeOH (1 mL) was added sodium acetate (43.9 mg, 0.53 mmol) and aniline (49.0 µL, 0.53 mmol). The reaction mixture was refluxed for 2 h and then the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, 75 : 25) afforded the title compound **93** as a yellow solid (40.1 mg, 0.20 mmol) in 51% yield.

δ_H (600 MHz, $CDCl_3$) 7.42-7.39 (2H, m, 2 x C*H*-Ar), 7.24 (1H, br s, NH), 7.17-7.14 (3H, m, 3 x C*H*-Ar), 5.51 (1H, s, C*H*-2), 3.05 (3H, s, C*H*$_3$-5); δ_C (150 MHz, $CDCl_3$) 172.9 (C=O), 168.4 (C=O), 142.6 (C), 138.5 (C), 129.9 (2 x CH), 124.6 (CH), 118.8 (2 x CH-Ar), 89.2 (CH), 23.9 (CH_3); IR 3292 (m), 1693 (s), 1614 (m), 1448 (m), 700 (m); MS (EI+) *m/z*, (relative intensity): 203 ([M]$^+$, 100), 94 (21). Exact mass calcd. for $[C_{11}H_{10}N_2O_2]^+$: 202.0737. Measured: 202.0732 (EI+); m.p.: 190 °C.

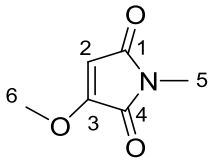
94. 3-Bromo-4-phenylamino-pyrrole-2,5-dione



To a mixture of 2,3-dibromomaleimide (100 mg, 0.39 mmol) and sodium acetate (32.4 mg, 0.39 mmol) in EtOH (2 mL) was added aniline (31.7 µL, 0.39 mmol). The reaction mixture was refluxed for 2 h and then the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, 75 : 25) afforded the title compound **94** as an orange solid (104 mg, 0.39 mmo) in 68% yield.

δ_H (500 Hz, $CDCl_3$) 7.42-7.38 (2H, m, 2 x C*H*-Ar), 7.29-7.26 (1H, m, C*H*-8), 7.22-7.20 (2H, m, 2 x C*H*-Ar), 7.09 (1H, br s, N*H*); δ_C (150 Hz, $CDCl_3$) 166.9 (C=O), 166.1 (C=O), 140.5 (C), 134.9 (C), 128.9 (2 x CH), 126.8 (CH), 124.7 (2 x CH), 81.7 (C); IR 3282 (m), 1715 (s), 1641 (s), 1614 (m); MS (EI+) *m/z*, (relative intensity): 268 ($[^{81}M]^+$, 98), 266 ($[^{79}M]^+$, 98), 144 (100). Exact mass calcd. for $[C_{10}H_7{}^{79}BrN_2O_2]^+$: 265.9685. Measured: 265.9679 (EI+); m.p.: 172 °C.

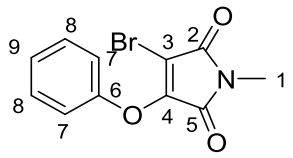
95. 3-Methoxy-1-methyl-pyrrole-2,5-dione[226]



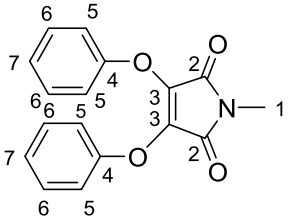
To a solution of bromomaleimide **57** (100 mg, 0.53 mmol) in MeOH (1.5 mL) was added sodium methoxide (42.6 mg, 0.78 mmol) and the resulting reaction mixture was stirred at room temperature for 10 min. The mixture was cooled to 0 - 4 °C, maintained at this temperature for a further 24 h and then the solvent was evaporated *in vacuo*. The crude mixture was redissolved in CH_2Cl_2 (10 mL) and washed with water (10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL) and the combined organic layers were dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 80 : 20 to 65 : 35) afforded the title compound **95** as a white solid (18.6 mg, 0.13 mmol) in 25% yield.

δ_H (600 MHz, $CDCl_3$) 5.40 (1H, s, C*H*-2), 3.93 (3H, s, CH_3-6), 2.99 (3H, s, CH_3-5); δ_C (150 Hz, $CDCl_3$) 170.3 (C=O), 165.8 (C=O), 161.2 (C), 96.3 (CH), 58.9 (CH_3), 23.6 (CH_3); IR 3112 (m), 1708 (s), 1634 (s), 1449 (s), 1324 (m), 957.1 (m), 818 (m); MS (EI+) *m/z*, (relative intensity): 141 ($[M]^+$, 72), 112 (100). Exact mass calcd. for $[C_6H_7NO_3]^+$: 141.0420. Measured: 141.0426 (EI+); m.p.: 124 °C (lit. m.p.[227]: 129 - 130 °C).

99. 3-Bromo-1-methyl-4-phenoxy-pyrrole-2,5-dione



100. 1-Methyl-3,4-diphenoxy-pyrrole-2,5-dione



A solution of phenol (147 mg, 1.56 mmol) in dry dioxane (2 mL) was added dropwise to a solution of potassium *tert*-butoxide (213 mg, 1.85 mmol) in dry dioxane (4 mL). 18-crown-6 (491 mg, 1.85 mmol) was added and the reaction mixture was stirred at room temperature for 15 min. The resulting mixture was added to a solution of *N*-methyl 2,3-dibromomaleimide (200 mg, 0.74 mmol) in dry dioxane (2.5 mL) over 30 min, and the stirring continued at room temperature for 48 h. After this time, the solvent was evaporated *in vacuo* and the resulting crude was redissolved in EtOAc. The organic layer was washed with 1 M NaOH (3 x 5 mL), brine (5 mL), dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, 80 : 20) afforded **99** as a white solid (45.1 mg, 0.16 mmol) in 22% yield. Further elution afforded **100** as a white solid (65.4 mg, 0.22 mmol) in 30% yield.

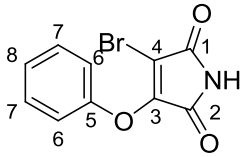
Compound 99:

δ_H (600 MHz, $CDCl_3$) 7.40-7.38 (2H, m, 2 x C*H*-Ar), 7.29-7.27 (1H, m, C*H*-9), 7.14-7.12 (2H, m, 2 x C*H*-Ar), 3.09 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 165.4 (C=O), 164.3 (C=O), 153.4 (C), 152.3 (C), 129.8 (2 x CH), 126.3 (CH), 119.7 (2 x CH), 98.3 (C), 24.7 (CH_3); IR 2927 (w), 1727 (s), 1649 (m), 1452 (m), 1197 (m); MS (CI+) *m/z* (relative intensity): 284 ($[^{81}M+H]^+$, 100), 282 ($[^{79}M+H]^+$, 100), 74 (14); Exact mass calcd. for $[C_{11}H_8NO_3{}^{79}Br]^+$+H: 281.9766 (CI+). Measured: 281.9764 (CI+); m.p.: 124 °C.

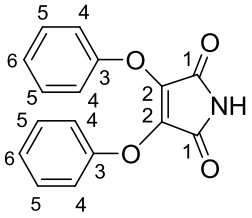
Compound 100:

δ_H (600 MHz, $CDCl_3$) 7.18-7.16 (4H, m, 4 x C*H*-Ar), 7.05-7.02 (2H, m, 2 x C*H*-7), 6.84-6.82 (4H, m, 4 x C*H*-Ar), 3.08 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 165.6 (2 x C=O), 154.9 (2 x C), 133.3 (2 x C), 129.5 (4 x CH), 124.6 (2 x CH), 117.3 (4 x CH), 23.8 (CH_3); IR 2923 (w), 1717 (s), 1683 (m), 1488 (m), 1445 (m), 1334 (m), 1232 (m), 1086 (m); MS (EI+) *m/z* (relative intensity): 295 ($[M]^+$, 35), 119 (33). Exact mass calcd. for $[C_{17}H_{13}NO_4]^+$: 295.0839. Measured: 295.0841; m.p. 103 - 105 °C.

97. 3-Bromo-4-phenoxy-pyrrole-2,5-dione



98. 3,4-Diphenoxy-pyrrole-2,5-dione



To a solution of phenol (244 mg, 2.59 mmol) in dry dioxane (2 mL) potassium *tert*-butoxide (31 mg, 2.95 mmol) in dry dioxane (4 mL) was added dropwise and the resulting solution was stirred for 10 min at room temperature. The resulting mixture was added dropwise to a solution of 2,3-dibromomaleimide (300 mg, 1.18 mmol) in dry dioxane (2 mL) and the mixture stirred at room temperature for 59 h. After this time, the solvent was evaporated *in vacuo.* The crude mixture was redissolved in EtOAc (20 mL), washed with water (10 mL), brine (10 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : Et_2O, gradient elution from 90 : 10 to 50 : 50) afforded the title compound **97** as a white solid (88.5 mg, 0.33 mmol) in 28% yield. Further elution afforded the title compound **98** as a yellow solid (7.2 mg, 0.02 mmol) in 2% yield

Compound 97:

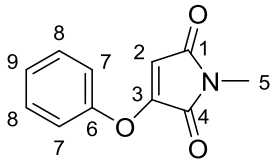
δ_H (600 MHz, $CDCl_3$) 7.95 (1H, br s, N*H*), 7.40 (2H, t, *J* = 8.3 Hz, 2 x C*H*-Ar), 7.30-7.27 (1H, m, C*H*-8), 7.15-7.13 (2H, m, 2 x C*H*-Ar); δ_C (150 MHz, $CDCl_3$) 164.4 (C=O), 163.5 (C=O), 153.2 (C), 152.4 (C), 129.9 (2 x CH), 126.4 (CH), 119.7 (2 x

CH), 99.4 (C); IR 3221 (br), 1719 (s), 1641 (s), 1488 (s), 1275 (s); MS (CI+) *m/z,* (relative intensity): 270 ($[^{81}M+H]^+$,97), 268 ($[^{79}M+H]^+$, 100). Exact mass calcd. for $[C_{10}H_6{}^{79}BrNO_3]^+$+H: 267.9609. Measured: 267.9612 (CI+); m.p.: 130 °C.

Compound 98:

δ_H (600 MHz, $CDCl_3$) 7.20-7.17 (4H, m, 4 x C*H*-Ar), 7.13 (1H, br s, N*H*), 7.06-7.03 (2H, m, 2 x C*H*-6), 6.85-6.83 (4H, m, 4 x C*H*-Ar); δ_C (150 MHz, $CDCl_3$) 164.4 (2 x C=O), 154.7 (2 x C), 133.9 (2 x C), 129.6 (4 x CH), 124.7 (2 x CH), 117.3 (4 x CH); IR 3276 (br), 1726 (s), 1590 (m), 1489 (s), 1339 (s), 1220 (m); MS (EI+) *m/z,* (relative intensity): 281 ($[M]^+$, 75); Exact mass calcd. for $[C_{16}H_{11}NO_4]^+$: 281.0683. Measured: 281.0682 (EI+); m.p.: 128 °C.

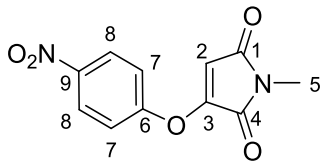
101. 1-Methyl-3-phenoxy-pyrrole-2,5-dione



To a solution of phenol (207 mg, 2.20 mmol) in dry dioxane (1 mL) potassium *tert*-butoxide (264 mg, 2.31 mmol) in dry dioxane (3 mL) was added dropwise and the solution was stirred for 15 min at room temperature. The resulting mixture was added dropwise to a solution of bromomaleimide **57** (400 mg, 2.10 mmol) in dry dioxane (1 mL) and the stirring was continued at room temperature for another 30 min. After this time, the solvent was evaporated *in vacuo*. Water (10 mL) was added and the crude product was extracted with EtOAc (3 x 30 mL), washed with brine (10 mL), dried ($MgSO_4$), filtered and the organic solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 80 : 20) afforded the title compound **101** as a white solid (321 mg, 1.58 mmol) in 75% yield.

δ_H (600 MHz, $CDCl_3$) 7.46-7.43 (2H, m, 2 x C*H*-Ar), 7.32-7.30 (1H, m, C*H*-9), 7.18-7.18 (2H, 2 x C*H*-Ar), 5.27 (1H, s, C*H*-2), 3.05 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 170.1 (C=O), 165.8 (C=O), 159.6 (C), 153.9 (C), 130.4 (2 x CH), 126.9 (CH), 119.9 (2 x CH), 99.4 (CH), 23.7 (CH_3); IR 1714 (s), 1637 (m), 1313 (m), 1220 (w); MS (EI+) *m/z*, (relative intensity): 203 ($[M]^+$, 100), 94 (21). Exact mass calcd. for $[C_{11}H_9NO_3]^+$: 203.0577. Measured: 203.0579 (EI+); m.p.: 63 °C.

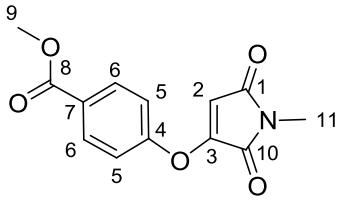
103. 1-Methyl-3-(4-nitrophenoxy)-pyrrole-2,5-dione



To a solution of 4-nitrophenol (155 mg, 1.10 mmol) in dry dioxane (1 mL) a mixture of potassium *tert*-butoxide (132 mg, 1.16 mmol) in dry dioxane (4 mL) and 18-crown ether (306 mg, 1.16 mmol) was added dropwise and the solution was stirred for 15 min. The resulting mixture was then added dropwise to a solution of bromomaleimide **57** (200 mg, 1.05 mmol) in dry dioxane (1 mL) and the stirring continued for another 20 h. After this time, the solvent was evaporated *in vacuo*. The resulting crude was redissolved in EtOAc (10 mL), washed with 1 M aq. NaOH (5 mL), extracted with EtOAc (3 x 10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded the title compound **103** as a white solid (114 mg, 0.46 mmol) in 44% yield.

δ_H (600 MHz, $CDCl_3$) 8.37-8.35 (2H, m, 2 x C*H*-Ar), 7.39-7.37 (2H, m, 2 x C*H*-Ar), 5.49 (1H, s, C*H*-2), 3.07 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 169.1 (C=O), 165.0 (C=O), 158.2 (C), 157.6 (C), 145.9 (C), 126.4 (2 x CH), 120.7 (2 x CH), 101.5 (CH), 23.9 (CH_3); IR 1713 (s), 1635 (m), 1588 (m), 1522 (m), 1350 (m), 1231 (w); MS (EI+) *m/z*, (relative intensity): 248 ($[M]^+$, 100), 150 (16). Exact mass calcd. for $[C_{11}H_8N_2O_5]^+$: 248.0428. Measured: 248.0435 (EI+); m.p.: 123 - 124 °C.

104. Methyl 4-(1-methyl-pyrrole-2,5-dione-3-yloxy)benzoate

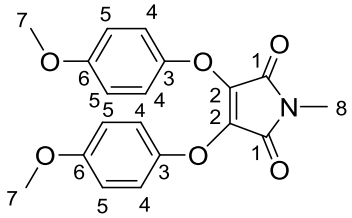


To a solution of methyl 4-hydroxybenzoate (170 mg, 1.10 mmol) in dry dioxane (1 mL) a mixture of potassium *tert*-butoxide (132 mg, 1.16 mmol) and 18-crown-6 ether (306 mg, 1.16 mmol) in dry dioxane (4 mL) was added dropwise and the resulting solution was stirred for 15 min at room temperature. The resulting mixture was

added dropwise to a solution of bromomaleimide **57** (200 mg, 1.05 mmol) in dry dioxane (1 mL) and stirred at room temperature for 19 h. After this time, the solvent was evaporated *in vacuo*, washed with 1 M aq. NaOH, extracted with EtOAc (3 x 30 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded the title compound **104** as yellow solid (196 mg, 0.75 mmol) in a 72% yield.

δ_H (600 MHz, $CDCl_3$) 8.15-8.13 (2H, m, 2 x C*H*-Ar), 7.27-7.25 (2H, m, 2 x C*H*-Ar), 5.37 (1H, s, C*H*-2), 3.94 (3H, s, CH_3-9), 3.06 (3H, s, CH_3-11); δ_C (150 MHz, $CDCl_3$) 169.7 (C), 165.9 (C), 165.4 (C), 158.5 (C), 157.2 (C), 132.2 (2 x CH), 128.9 (C), 119.9 (2 x CH), 100.3 (CH), 52.6 (CH_3), 23.9 (CH_3); IR 1731 (s), 1713 (s), 1638 (m), 1600 (m), 1440 (m), 1285 (m), 1105 (m); MS (EI+) *m/z*, (relative intensity): 261 ($[M]^+$, 100), 86 (20). Exact mass calcd. for $[C_{13}H_{11}NO_5]^+$: 261.0632. Measured: 261.0636 (EI+); m.p.: 149 °C.

105. 3,4-*bis*(4-Methoxyphenoxy)-1-methyl-pyrrole-2,5-dione

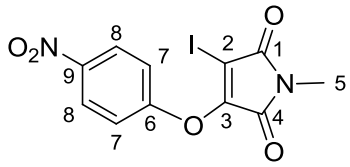


A solution of 4-methoxyphenol (199 mg, 1.59 mmol) in dry dioxane (1.9 mL) was added dropwise to a solution of potassium *tert*-butoxide (206 mg, 1.85 mmol) in dry dioxane (4.3 mL). The reaction mixture was stirred for 15 min then added to a solution of *N*-methyl 2,3-dibromomaleimide (200 mg, 0.74 mmol) in dry dioxane (2.5 mL) over 30 min. The resulting mixture was stirred at room temperature for 20 h, then concentrated *in vacuo* and redissolved in EtOAc (30 mL). The organic layer was washed with 1 M NaOH (10 mL), with brine (10 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo.* Purification by flash chromatography (PE : EtOAc, gradient elution from 95 : 5 to 40 : 60) afforded the title compound **105** as a yellow solid (33.1 mg, 0.10 mmol) in 13% yield.

δ_H (600 MHz, $CDCl_3$) 6.80-6.78 (4H, m, 4 x C*H*-Ar), 6.71-6.68 (4H, m, 4 x C*H*-Ar), 3.76 (6H, s, 2 x CH_3-7), 3.04 (3H, s, CH_3-8); δ_C (150 MHz, $CDCl_3$) 165.8 (2 x C=O),

156.5 (C), 148.9 (C), 133.5 (C), 118.4 (4 x CH), 114.4 (4 x CH), 55.7 (2 x CH_3), 23.7 (CH_3); IR 2955 (w), 1717 (s), 1504 (s), 1227 (m); MS (EI+) *m/z*, (relative intensity): 355 ($[M]^+$, 100), 123 (83). Exact mass calcd. for $[C_{19}H_{17}NO_6]^+$: 355.1056. Measured: 355.1056 (EI+); m.p.: 79 °C.

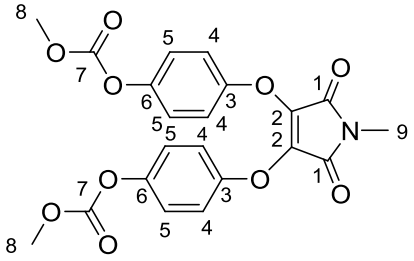
106. 3-Iodo-1-methyl-4-(4-nitrophenoxy)-pyrrole-2,5-dione



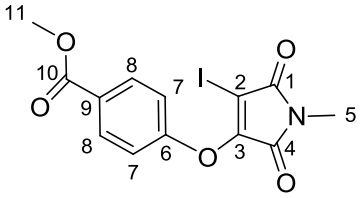
A solution of 4-nitrophenol (89.4 mg, 0.64 mmol) in dry dioxane (0.5 mL) was added dropwise to a solution of potassium *tert*-butoxide (89.4 mg, 0.72 mmol) and 18-crown-6 ether (191 mg, 0.72 mmol) in dry dioxane (3 mL). The reaction mixture was stirred for 15 min then added over 30 min to a solution of diiodomaleimide **90** (200 mg, 0.55 mmol) in dry dioxane (1 mL). The mixture was stirred at room temperature for 64 h, then concentrated *in vacuo* and redissolved in EtOAc (30 mL). The organic layer was washed with 1 M aq. NaOH (10 mL), brine (10 mL), dried ($MgSO_4$), filtered and the solvent was evaporated in *vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 60 : 40) afforded the title compound **106** as a yellow solid (50.7 mg, 0.14 mmol) in 47% yield.

δ_H (600 MHz, $CDCl_3$) 8.31-8.29 (2H, m, 2 x C*H*-Ar), 7.24-7.22 (2H, m, 2 x C*H*-Ar), 3.11 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 165.3 (C=O), 163.9 (C=O), 158.2 (C), 158.1 (C), 145.2 (C), 125.9 (2 x CH), 119.5 (2 x CH), 79.1 (C), 25.2 (CH_3); IR 1714 (s), 1642 (m), 1586 (m), 1346 (m), 1274 (m); MS (EI+) *m/z*, (relative intensity): 374 ($[M]^+$, 100), 168 (56). Exact mass calcd. for $[C_{11}H_7O_5N_2I]^+$: 373.9394; Measured: 373.9391 (EI+); m.p.: 117 °C.

107. Dimethyl 4,4'-(1-methyl-3,4 diyl(*bis*(oxy)*bis*(4,1-phenylene) dicarbonate) - pyrrole-2,5-dione) x



108. Methyl 4-(4-iodo-1-methyl-pyrrole-2,5-dione-3-yloxy)benzoate (43)



A solution of methyl 4-hydroxybenzoate (186 mg, 1.21 mmol) in dry dioxane (1 mL) was added dropwise to a solution of potassium *tert*-butoxide (206 mg, 1.85 mmol) and 18-crown ether (363 mg, 1.37 mmol) in dry dioxane (4 mL). The reaction mixture was stirred at room temperature for 15 min then added to a solution of diiodomaleimide **90** (200 mg, 0.55 mmol) in dry dioxane (1 mL) over 30 min. The mixture was stirred at room temperature for 24 h, then concentrated *in vacuo* and dissolved in EtOAc (30 mL). The organic layer was washed with 1 M NaOH (10 mL), brine (10 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo.* Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 60 : 40) afforded the title compound **107** as a white solid (20.1 mg, 0.05 mmol) in 10% yield. Further elution gave the title compound **108** as a white solid (29.6 mg, 0.07 mmol) in 13% yield.

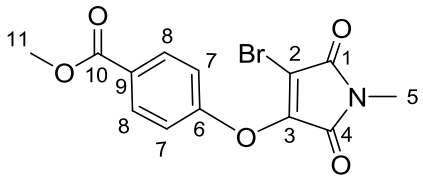
Compound 107:

δ_H (600 MHz, $CDCl_3$) 7.90-7.89 (4H, m, 4 x C*H*-Ar), 6.91-6.89 (4H, m, 4 x C*H*-Ar), 3.89 (6H, s, 2 x CH_3-8), 3.18 (3H, s, CH_3-9); δ_C (150 MHz, $CDCl_3$) 166.1 (2 x C=O), 164.7 (2 x C=O), 157.9 (2 x C), 133.1 (2 x C), 131.5 (4 x CH), 126.8 (2 x C), 117.1 (4 x CH), 52.4 (2 x CH_3), 23.9 (CH_3); IR 1715 (s), 1602 (m), 1436 (m), 1279 (s); MS (EI+) *m/z*, (relative intensity): 411 ($[M]^+$, 100), 380 (50). Exact mass calcd. for $[C_{21}H_{17}NO_8]^+$: 411.0948. Measured: 411.0938 (EI+); m.p.: 120 °C.

Compound 108:

δ_H (600 MHz, $CDCl_3$) 8.12-8.10 (2H, m, 2 x C*H*-Ar), 7.19-7.16 (2H, m, 2 x C*H*-Ar), 3.95 (3H, s, CH_3-11), 3.12 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 166.1 (C=O), 165.9 (C=O), 164.3 (C=O), 158.4 (C), 156.8 (C), 113.7 (2 x CH), 127.9 (C), 119.4 (2 x CH), 75.7 (C), 52.5 (CH_3), 25.0 (CH_3); IR 1715 (s), 1597 (m), 1436 (m), 1276 (s); MS (EI+) *m/z*, (relative intensity): 387 ($[M]^+$, 100), 356 (23). Exact mass calcd. for $[C_{13}H_{10}NO_5I]^+$: 386.9598. Measured: 386.9599 (EI+); m.p.: 92 - 94 °C.

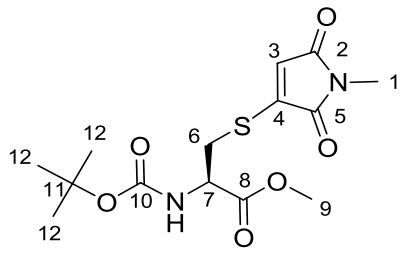
109. Methyl 4-(4-bromo-1-methyl-pyrrole-2,5-dione-3-yloxy)benzoate



A solution of methyl 4-hydroxybenzoate (124 mg, 0.81 mmol) in dry dioxane (1 mL) was added dropwise to a solution of potassium *tert*-butoxide (90.9 mg, 0.81 mmol) and 18-crown ether (213 mg, 0.81 mmol) in dry dioxane (4 mL). The reaction mixture was stirred for 15 min then added to a solution of *N*-methyl-2,3-dibromomaleimide (200 mg, 0.74 mmol) in dry dioxane (1 mL) over 30 min. The mixture was stirred at room temperature for 10 h, then concentrated *in vacuo* and redissolved in EtOAc (30 mL). The organic layer was washed with 1 M aq. NaOH (10 mL), brine (10 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 80 : 20 to 70 : 30) afforded the title compound **109** as a yellow solid (159 mg, 0.47 mmol) in 63% yield.

δ_H (600 MHz, $CDCl_3$) 8.10-8.07 (2H, m, 2 x C*H*-Ar), 7.17- 7.15 (2H, m, 2 x C*H*-Ar), 3.92 (3H, s, CH_3-11), 3.09 (3H, s, CH_3-5); δ_C (150 MHz, $CDCl_3$) 166.1 (C=O), 164.8 (C=O), 163.9 (C=O), 156.9 (C), 151.7 (C), 131.7 (2 x CH), 127.9 (C), 119.1 (2 x CH-Ar), 101.4 (C), 52.5 (CH_3), 24.8 (CH_3); IR 1719 (s), 1649 (m), 1599 (m), 1436 (m), 1277 (s); MS (EI+) *m/z*, (relative intensity): 341 ($[^{81}M]^+$, 100), 339 ($[^{79}M]^+$, 100), 310 (56), 308 (55). Exact mass calcd. for $[C_{13}H_{10}NO_5{}^{79}Br]^+$: 338.9737. Measured: 338.9342 (EI+); m.p.: 132 - 133 °C.

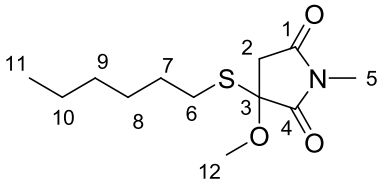
110. (R)-Methyl 2-(*tert*-butoxycarbonylamino)-3-(1-methyl-pyrrole-2,5-dione-3-ylthio)propanoate[138]



To a solution of *N*-Boc-Cys-OMe (28.3 mg, 0.11 mmol) and sodium acetate (10.1 mg, 0.12 mmol) in MeOH (2.7 mL) a solution of sulphoxymaleimide **66** (29.3 mg, 0.12 mmol) in MeOH (2.7 mL) was added over 15 min. The reaction mixture turned pink, then green. The solvent was evaporated *in vacuo.* Purification by flash chromatography (PE : EtOAc, gradient elution from 70 : 30 to 40 : 60) afforded the title compound **110** as a white solid (18.0 mg, 0.06 mmol) in 52% yield.

δ_H (600 MHz, $CDCl_3$) 6.26 (s, 1H, C*H*-3), 5.37 (1H, d, *J* = 6.9 Hz, N*H*), 4.67 (2H, dd, *J* = 11.9, 6.7 Hz, C*H*-7), 3.79 (3H, s, CH_3-9), 3.47 (1H, dd, *J* = 13.8, 5.1 Hz, C*H*H-6), 3.35 (1H, dd, *J* = 13.8, 5.1 Hz, CH*H*-6), 3.00 (3H, s, CH_3-1), 1.43 (9H, s, 3 x CH_3-12); δ_C (150 Hz, $CDCl_3$) 170.2 (C=O), 169.5 (C=O), 167.9 (C=O), 155.1 (C=O), 150.0 (C), 118.7 (CH), 81.0 (C), 53.3 (CH_3), 52.8 (CH_3), 33.9 (CH_2), 28.4 (3 x CH_3); IR 1703 (s), 1560 (w), 1441 (m), 1378 (w), 1253 (m); m.p.: 143 °C (lit. m.p.[138]: 101 - 103 °C).

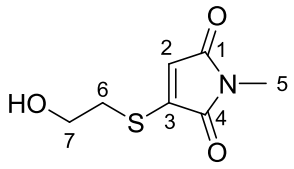
116. 3-Hexylthio-3-methoxy-1-methyl-pyrrolidine-2,5-dione



To a solution of methoxymaleimide **95** (6.3 mg, 0.04 mmol) and sodium acetate (7.30 mg, 0.09 mmol) in MeOH (6 mL) *n*-hexanethiol (63.0 µL, 0.45 mmol) in MeOH (4 mL) was added dropwise and the resulting mixture was stirred at room temperature. After 72 h of the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 60 : 40) afforded the title compound **116** as a transparent oil (10.4 mg, 0.04 mmol) in 90% yield.

δ_H (600 MHz, $CDCl_3$) 3.44 (3H, s, C*H*$_3$-12), 3.02 (3H, s, C*H*$_3$-5), 2.99 (1H, d, *J* = 17.9 Hz, CH*H*-2), 2.81 (1H, ddd, *J* = 12.3, 8.2, 6.9 Hz, CH*H*-6), 2.74 (1H, ddd, *J* = 12.3, 8.2, 6.9 Hz, C*H*H-6), 2.71 (1H, d, *J* = 17.8, CH*H*-2), 1.59-1.52 (2H, m, C*H*$_2$-7), 1.39-1.34 (2H,m, C*H*$_2$-8), 1.31-1.24 (4H, m, C*H*$_2$-9 and C*H*$_2$-10), 0.87 (3H, t, *J* =7.0 Hz, C*H*$_3$-11); δ_C (150 MHz, $CDCl_3$) 173.1 (C=O), 172.1 (C=O), 87.8 (C), 51.8 (CH_3), 42.1 (CH_2), 31.4 (CH_2), 29.0 (CH_2), 28.8 (CH_2), 28.6 (CH_2), 25.1 (CH_3), 22.6 (CH_2), 14.1 (CH_3), one CH_2 missing due to overlap; IR 2927 (m), 1710 (s), 1434 (m), 1284 (m); MS (CI+) *m/z* (relative intensity): 260 ([M]$^+$, 51), 228 (100). Exact mass calcd. for $[C_{12}H_{22}NO_3S]^+$+H: 260.1320. Measured: 260.1316 (EI+).

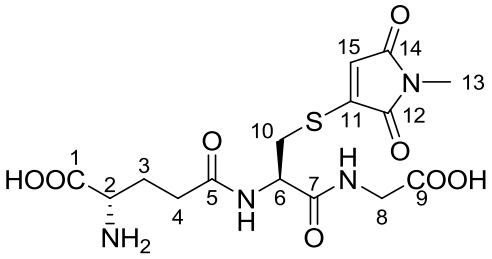
125. 3-(2-Hydroxyethylthio)-1-methyl-pyrrole-2,5-dione



To a stirred solution of bromomaleimide **57** (100 mg, 0.53 mmol) and sodium acetate (93.2 mg, 0.53 mmol) in MeOH (60 mL) was added 2-mercaptoethanol (0.035 mL, 0.51 mmol) at room temperature. After 5 min, the solvent was evaporated *in vacuo.* Purification by flash chromatography (PE: EtOAc, gradient elution from 60 : 40 to 50 : 50) afforded the title compound **125** as a yellow solid (80 mg, 0.43 mmol) in 84%.

δ_H (500 MHz, $CDCl_3$) 6.17 (1H, s, C*H*-2), 3.95 (2H, q, *J* = 5.9 Hz, C*H*-7), 3.16 (2H, t, *J* = 5.8 Hz, C*H*$_2$-6), 3.02 (3H, s, C*H*$_3$-5), 1.87 (1H, t, *J* = 5.7 Hz, OH); δ_C (150 MHz, $CDCl_3$) 169.7 (C=O), 168.1 (C=O), 150.6 (C), 118.4 (CH), 60.2 (CH_2), 34.5 (CH_2), 24.2 (CH_3); IR 3446 (br), 1690 (s), 1555 (m), 1440 (m), 1384 (m), 973 (s); MS (EI+) *m/z*, (relative intensity): 187 ([M]$^+$, 7), 187 (100). Exact mass calcd. for $[C_7H_9NO_3S]^+$: 187.0298; Measured: 187.0300 (EI+); m.p.: 63 °C.

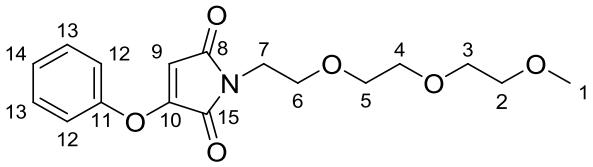
126. (R)-2-amino-5-((S)-1-(carboxymethylamino)-3-(1-methyl-pyrrole-2,5-dione-3-ylthio)-1-oxopropan-2-ylamino)-5-oxopentanoic acid



A solution of reduced glutathione (7.68 mg, 0.25 mmol) in 2.5 mL pH 6 buffer (50 mM sodium phosphate) was added dropwise to a solution of bromomaleimide **57** (4.75 mg, 0.25 mmol) in pH 6 buffer (50 mM sodium phosphate, 20% CH_3CN). The reaction mixture was stirred at room temperature for 5 min. Part of the crude was purified by HP-LC (H_2O : CH_3CN = 95 : 5 to 5 : 95) to afford the title compound **126** as yellow oil.

δ_H (600 MHz, MeOD) 6.56 (1H, s, C*H*-15), 4.78 (1H, dd, *J* = 8.1, 5.9 Hz, C*H*-6), 4.03 (1H, t, *J* = 6.3 Hz, C*H*-2), 3.95 (2H, s, CH_2-8), 3.49 (1H, dd, *J* = 13.6 , 5.9, Hz, CH*H*-10), 3.26 (1H, dd, *J* = 13.6, 8.2 Hz, C*H*H-10), 2.97 (3H, s, CH_3-13), 2.57 (2H, t, *J* = 6.9 Hz, CH_2-4), 2.25-2.13 (2H, m, CH_2-3); δ_C (150 MHz, $CDCl_3$) 174.5 (C=O), 172.8 (C=O), 171.9 (C=O), 171.5 (C=O), 171.3 (C=O), 169.5 (C=O), 151.2 (C), 120.0 (CH), 53.4 (CH), 52.6 (CH), 41.8 (CH_2), 33.8 (CH_2), 32.2 (CH_2), 26.9 (CH_2), 24.1 (CH_3); MS (CI+) *m/z*, (relative intensity): 439 ($[M+Na]^+$, 77), 287 (100). Exact mass calcd. for $[C_{15}H_{20}N_4O_8S]^+$+Na: 439.0897; Measured: 439.0900 (EI+).

133. 1-(2-(2-(2-Methoxyethoxy)ethoxy)ethyl)-3-phenoxy-pyrrole-2,5-dione



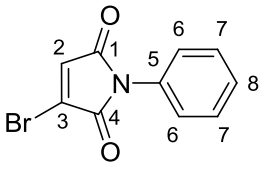
Method 1: A solution of PEG amine **140** (32.8 mg, 0.10 mmol) in CH_2Cl_2 (0.5 mL) was added to a solution of *N*-methoxycarbonyl phenoxymaleimide **151** (21.0 mg, 0.08 mmol) in CH_2Cl_2 (0.5 mL) and the resulting mixture was stirred at room temperature for 15 h. After this time, the solvent was evaporated *in vacuo.* Purification by flash chromatography (CH_2Cl_2 : MeOH, gradient elution from 97 : 3 to

95 : 5) afforded the title compound **146** as a transparent oil (7.60 mg, 0.02 mmol) in 25% yield.

Method 2: To a solution of phenol (48.0 mg, 0.51 mmol) in dry dioxane (0.5 mL) potassium *tert*-butoxide (57.2 mg, 0.51 mmol) in dry dioxane (2.5 mL) was added dropwise. The resulting mixture was stirred for 15 min at room temperature and then added dropwise to a solution of *N*-PEG bromomaleimide **145** (85.8 mg, 0.26 mmol) in dry dioxane (1 mL). The mixture was stirred at room temperature for 1 h and then the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 70 : 30 to 40 : 60) afforded the title compound **146** as a transparent oil (39.6 mg, 0.12. mmol) in 46% yield.

δ_H (600 MHz, $CDCl_3$) 7.46-7.43 (2H, m, 2 x C*H*-Ar), 7.32-7.30 (1H, t, *J* = 6.0 Hz, C*H*-14), 7.20-7.18 (2H, d, *J* = 12 Hz, 2 x C*H*-Ar), 5.27 (1H, s, C*H*-9), 3.76-3.74 (2H, t, *J* = 6 Hz, CH_2), 3.68-3.63 (8H, m, 4 x CH_2), 3.55-3.54 (2H, m, CH_2), 3.37 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 169.9 (C=O), 165.6 (C=O), 159.5 (C), 153.9 (C), 130.4 (2 x CH), 126.9 (CH), 119.9 (2 x CH), 99.4 (CH), 72.0 (CH_2), 70.72 (CH_2), 70.70 (CH_2), 70.1 (CH_2), 68.1 (CH_2), 59.2 (CH_3), 37.1 (CH_2); IR 2917 (br), 1715 (s), 1633 (s), 1312 (m); MS (CI+) m/z, (relative intensity): 336 ($[M+H]^+$, 98), 216 (100). Exact mass calcd. for $[C_{17}H_{21}NO_6]^+$+H: 336.1447. Measured: 336.1445 (CI+).

138. 3-Bromo-1-phenyl-pyrrole-2,5-dione[227]

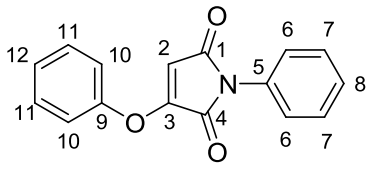


To a solution of *N*-phenyl maleimide **137** (400 mg, 2.31 mmol) in (3 mL) was added dropwise a solution of bromine (237 µL, 4.62 mmol) in chloroform (1 mL) at room temperature. The reaction mixture was refluxed for 2 h and then allowed to cool to room temperature. The reaction mixture was diluted with chloroform (10 mL), washed with sat. aq. $Na_2S_2O_3$ (5 mL), water (5 mL) and brine (5 mL). The crude product was dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo* to afford 636 mg of crude dibromosuccinimide as a yellow solid. Then, a part of the dibromosuccinimide (400 mg, 1.32 mmol) was dissolved in acetic acid (8 mL) and sodium acetate (325 mg, 3.96 mmol) was added. The reaction mixture was refluxed for 2 h and then the solvent was evaporated *in vacuo*. The crude residue was diluted

with ethyl acetate (30 mL) and washed with water (20 mL), brine (20 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (petroleum ether : ethyl acetate, gradient elution from 90 : 10 to 80 : 20) afforded the title compound **138** as a white solid (179.5 mg, 0.71mmol) in 54% yield.

δ_H (600 MHz, $CDCl_3$) 7.48 (2H, t, *J* = 7.8 Hz, 2 x C*H*-7), 7.41-7.38 (1H, m, C*H*-8), 7.34 (2H, d, *J* = 7.5 Hz, 2 x C*H*-6), 7.03 (1H, s, C*H*-2); δ_C (150 MHz, $CDCl_3$) 167.5 (C=O), 164.3 (C=O), 132.0 (CH), 131.9 (C), 131.1 (C), 129.4 (2 x CH), 128.5 (CH), 126.2 (2 x CH); IR 1711 (s), 1592 (m), 1504 (m), 1396 (s), 1148 (m); MS (EI+) *m/z,* (relative intensity): 253 ($[^{81}M]^+$, 100), 251 ($[^{79}M]^+$, 96), 86 (55), 84 (90). Exact mass calcd. for $[C_{10}H_6Br^{79}NO_2]^+$: 250.9582. Measured: 250.9582 (EI+); m.p.: 153 °C (lit. m.p.[229]: 152 °C).

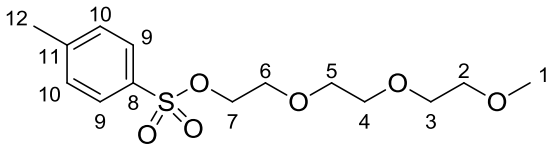
139. 3-Phenoxy-1-phenyl-pyrrole-2,5-dione



To a solution of phenol (22.3 mg, 0.24 mmol) in dry dioxane (0.5 mL) potassium *tert*-butoxide (26.9 mg, 0.24 mmol) in dry dioxane (2.5 mL) was added dropwise. The solution was left stirring for 15 min and then added dropwise to a solution of *N*-phenyl bromomaleimide **138** (50.0 mg, 0.20 mmol) in dry dioxane (1 mL). The resulting mixture was stirred at room temperature for 2.5 h and then the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : Et_2O, gradient elution from 90 : 10 to 80 : 20) afforded the title compound **139** as a yellow solid (18.8 mg, 0.07 mmol) in 46% yield.

δ_H (600 MHz, $CDCl_3$) 7.61-7.51 (4H, m, 4 x C*H*-Ar), 7.42-7.35 (4H, m, 4 x C*H*-Ar), 7.28-7.26 (2H, m, 2 x C*H*-Ar), 5.46 (1H, s, C*H*-2); δ_C (150 MHz, $CDCl_3$) 168.9 (C=O), 164.5 (C=O), 159.4 (C), 153.9 (C), 131.1 (C), 130.5 (2 x CH), 129.3 (2 x CH), 128.1 (CH), 127.1 (CH), 126.2 (2 x CH), 120.0 (2 x CH), 99.5 (CH); IR 1715 (s), 1634 (m), 1401 (m), 1201 (m); MS (ES+) *m/z,* (relative intensity): 266 ($[M+H]^+$, 42), 180 (100). Exact mass calcd. for $[C_{16}H_{11}NO_3]^+$+H: 266.0817. Measured: 266.0827 (ES+); m.p.: 198 - 201 °C.

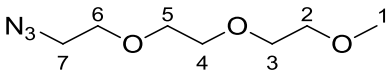
142. 2-(2-(2-Methoxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate[196]



Triethylene glycol monomethyl ether **141** (2.92 mL, 18.3 mmol) and triethylamine (3.80 mL, 27.4 mmol) were dissolved in dry CH_2Cl_2 (15 mL) and the solution was cooled to 0 °C. A solution of tosyl chloride (3.48 g, 18.3 mmol) in dry CH_2Cl_2 (5 mL) was then added dropwise. The mixture was stirred for 3 h at 0 °C and then allowed to warm to room temperature over for 14 h. After that, Et_2O (20 mL) was added and the resulting precipitate was filtered. The filtrate was concentrated *in vacuo*. Purification by flash column chromatography (Et_2O) afforded the title compound **142** as a colourless oil (4.57 g, 14.3 mmol) in 79% yield.

δ_H (600 MHz, $CDCl_3$) 7.80 (2H, d, *J* = 12.0 Hz, 2 x C*H*-Ar), 7.34 (2H, d, *J* = 12.0 Hz, 2 x C*H*-Ar), 4.16 (2H, t, *J* = 6.0 Hz, CH_2-7), 3.69 (2H, t, *J* = 6.0 Hz, CH_2-6), 3.61-3.59 (6H, m, 3 x CH_2), 3.54-3.52 (2H, m, CH_2), 3.37 (3H, s, CH_3-1), 2.45 (3H, s, CH_3-12); δ_C (150 MHz, $CDCl_3$) 144.9 (C), 133.1 (C), 129.9 (2 x CH), 128.1 (2 x CH), 72.0 (CH_2), 70.9 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 69.3 (CH_2), 68.8 (CH_2), 59.2 (CH_3), 21.8 (CH_3); IR 2881 (br), 1355 (m), 1189 (m), 1170 (s), 1099 (m); MS (CI+) *m/z,* (relative intensity): 319 ($[M+H]^+$, 80), 199 (65), 147 (65), 103 (100). Exact mass calcd. for $[C_{14}H_{22}O_6S]^+$+H: 319.1209. Measured: 319.1209 (CI+).

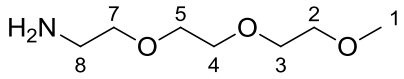
143. 1-Azido-2-(2-(2-methoxyethoxy)ethoxy)ethane[196]



To a solution of tosylated PEG **142** (1.90 g, 5.98 mol) in DMF (40 mL) was added sodium azide (0.96 g, 14.9 mmol). The reaction mixture was stirred for 20 h at 60 °C and then for 3 h at 120 °C. After this time, the mixture was allowed to cool to room temperature and then Et_2O (50 mL) was added. The organic phase was washed with sat. aq. LiCl (5 x 20 mL), brine (20 mL), dried (Na_2SO_4), filtered and the solvent was evaporated *in vacuo* to afford the title compound **143** as a brown oil (887 mg, 4.69 mmol) in 78% yield.

δ_H (600 MHz, $CDCl_3$) 3.68-3.65 (8H, m, 4 x CH_2), 3.55 (2H, t, *J* = 4.8 Hz, CH_2), 3.40-3.38 (5H, m, CH_3-1 and C*H*-2); δ_C (150 MHz, $CDCl_3$) 72.0 (CH_2), 70.81 (CH_2), 70.78 (CH_2), 70.74 (CH_2), 70.2 (CH_2), 59.2 (CH_3), 50.8 (CH_2); IR 2874 (br), 2098 (s), 1288 (br), 1104 (s); MS (CI+) *m/z,* (relative intensity): 190 ($[M+H]^+$, 20), 162 (65), 103 (100).

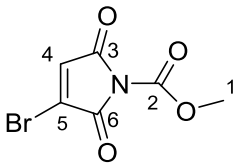
140. 2-(2-(2-Methoxyethoxy)ethoxy)ethanamine[196]



The azide PEG **143** (880 mg, 4.65 mmol) was dissolved in Et_2O (50 mL) and the solution cooled at 0 °C. Triphenyl phosphine (1.46 g, 5.58 mmol) was added and the mixture was stirred for 1 h at 0 °C and for another 1 h at room temperature. The reaction was quenched with water (20 mL) and the resulting mixture stirred vigorously for 4 h. Then, toluene (15 mL) was added and the mixture was stirred for 12 h. The layers were separated and the aq. layer was washed with toluene (10 mL) and evaporated *in vacuo* to afford the title compound **140** as a brown oil (506 mg, 3.10 mmol) in 67% yield.

δ_H (600 MHz, $CDCl_3$) 3.63-3.60 (6H, m, 3 x CH_2), 3.53-3.52 (2H, m, CH_2), 3.49 (2H, t, *J* = 4.8 Hz, CH_2-7), 3.35 (3H, s, CH_3-1), 2.85 (2H, t, *J* = 6.0 Hz, CH_2-8); δ_C (150 MHz, $CDCl_3$) 73.0 (CH_2), 71.9 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 70.3 (CH_2), 59.1 (CH_3), 41.7 (CH_2); IR 3379 (br), 2876 (br), 1570 (br), 1462 (br), 1304 (s), 1097 (s); MS (CI+) *m/z,* (relative intensity): 164 ($[M+H]^+$, 100), 88 (25). Exact mass calcd. for $[C_7H_{17}NO_3]^+$+H: 164.1287. Measured: 164.1289 (CI+).

144. Methyl 3-bromo-pyrrole-2,5-dione carboxylate

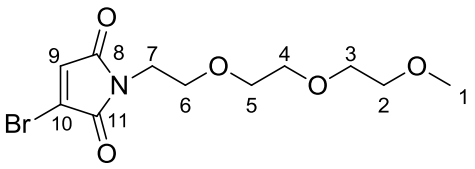


To a solution of bromomaleimide **22** (200 mg, 1.14 mmol) in THF (6 mL) were added a solution of methyl chloroformate (88.3 µL, 1.14 mmol) in THF (2 mL) and a solution of *N*-methylmorpholine (125 µL, 1.14 mmol) in THF (2 mL). The reaction mixture was stirred at room temperature for 30 min. After this time, CH_2Cl_2 (5 mL)

was added and the solution was washed with water (3 x 10 mL), dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo* to afford the title compound **144** as a pale pink solid (266 mg, 1.14 mmol) in 100% yield.

δ_H (600 MHz, $CDCl_3$) 7.04 (1H, s, C*H*-4), 4.01 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 163.5 (C=O), 160.9 (C=O), 147.6 (C=O), 133.3 (C), 133.1 (CH), 54.7 (CH_3); IR 1808 (m), 1763 (s), 1724 (S), 1603 (m), 1438 (m), 1320 (s), 1263 (s), 1070 (s); MS (EI+) *m/z,* (relative intensity): 235 ($[^{81}M]^+$, 13), 233 ($[^{79}M]^+$, 13). Exact mass calcd. for $[C_6H_4{}^{79}BrNO_4]^+$: 232.9318. Measured: 232.9323 (EI+); m.p.: 121 °C.

145. 3-Bromo-1-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-pyrrole-2,5-dione

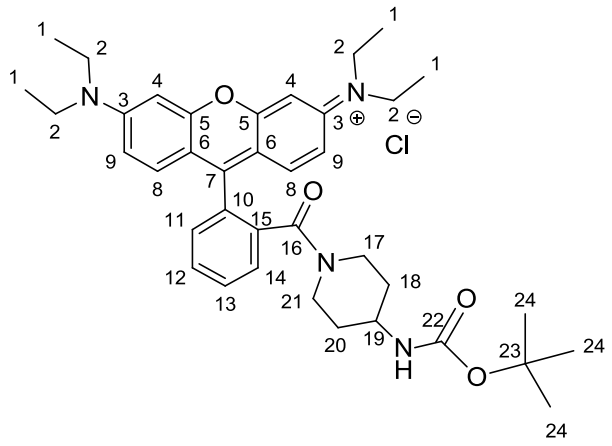


A solution of PEG amine **140** (157 mg, 0.96 mmol) in CH_2Cl_2 (2 mL) was added to a solution of *N*-methoxycarbonyl bromomaleimide **144** (224 mg, 0.96 mmol) in CH_2Cl_2 (8 mL) and stirred at room temperature for 1 h. After this time, the solvent was evaporated *in vacuo.* Purification by flash chromatography (CH_2Cl_2 : MeOH, gradient elution from 97 : 3 to 95 : 5) afforded the title compound **145** as a yellow oil (136.2 mg, 0.42 mmol) in 44%.

δ_H (600 MHz, $CDCl_3$) 6.86 (1H, s, C*H*-9), 3.77 (2H, t, *J* = 6.0 Hz, CH_2), 3.65 (2H, t, *J* = 6 Hz, CH_2), 3.63-3.59 (6H, m, 3 x CH_2), 3.53-3.52 (2H, m, CH_2), 3.37 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$)* 168.6 (C=O), 165.4 (C=O), 132.0 (CH), 131.5 (C), 72.0 (CH_2), 70.7 (CH_2), 70.2 (CH_2), 67.8 (CH_2), 59.2 (CH_3), 38.2 (CH_2); IR 2878 (m), 1715 (s), 1396 (s), 1105 (m); MS (CI+) *m/z,* (relative intensity): 324 ($[^{81}M+H]^+$, 70), 322 ($[^{79}M+H]^+$, 68). Exact mass calcd. for $[C_{11}H_{16}Br^{79}NO_5]^+$+H: 322.0290. Measured: 322.0298 (CI+).

* one CH_2 missing due to overlap.

148. *N*-(9-(2-(4-(*tert*-Butoxycarbonylamino)piperidine-1-carbonyl)phenyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium chloride[149]

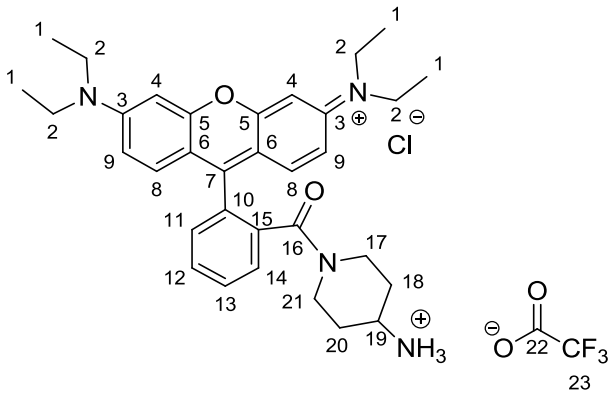


Rhodamine B (90.0 mg, 0.19 mmol) was dissolved in oxalyl chloride (18 mL) and the reaction mixture was stirred at room temperature for 24 h. After this time, the solvent was evaporated *in vacuo* and the residue was dissolved in CH_2Cl_2 (9 mL). This solution was then added dropwise to a stirred solution of piperidin-4-yl-carbamic acid *tert*-butyl ester (376 mg, 0.20 mmol) and Cs_2CO_3 (612 mg, 20.0 mmol) in CH_2Cl_2 (9 mL) and the resulting reaction mixture was stirred at room temperature for 24 h. After this time, the solvent was evaporated *in vacuo*. Purification by flash chromatography (CH_2Cl_2 : MeOH, gradient elution from 98 : 2 to 90 : 10) afforded the title compound **148** as a purple solid (81.0 mg, 0.12 mmol) in 64% yield.

δ_H (600 MHz, MeOD)* 7.78-7.74 (2H, m, C*H*-11 and C*H*-14), 7.66-7.65 (1H, m, C*H*-12 or C*H*-13), 7.51-7.49 (1H, m, C*H*-12 or C*H*-13), 7.27 (2H, dd, *J* = 9.4, 3.1 Hz, C*H*-8), 7.08-7.06 (2H, m, 2 x C*H*-9), 6.97 (2H, d, *J* = 2.5 Hz, 2 x C*H*-4), 4.08 (1H, d, *J* = 13.5 Hz, C*H*H), 3.70 (8H, q, *J* = 7.2 Hz, 4 x CH_2-2), 3.48-3.42 (1H, m, CH*H*), 3.05 (1H,br s,C*H*H), 2.68 (1H, br s, CH*H*), 1.75 (2H, t, *J* = 11.5 Hz,CH_2), 1.41 (9H, s, 3 x CH_3-24), 1.32 (12H, t, *J* = 7.4 Hz, 4 x CH_3-1), 1.19-1.10 (2H, m, CH_2); δ_C (150 MHz, MeOD) 169.3 (2 x C), 159.3 (2 x C), 157.2 (C), 157.0 (C), 137.0 (C), 133.2 (CH), 132.0 (CH), 131.7 (CH), 131.3 (CH), 131.1 (2 x CH), 128.6 (CH), 115.4 (CH), 115.2 (CH), 113.4 (C), 97.3 (2 x CH), 80.1 (C), 47.7 (2 x CH_2), 46.9 (4 x CH_2), 41.8 (2 x CH_2), 28.7 (3 x CH_3), 12.8 (4 x CH_3); IR 3370 (br), 2973 (br), 1623 (m), 1584 (s), 1409 (m), 1262 (s); MS (FAB+) *m/z,* (relative intensity): 625 ($[M]^+$, 15); Exact mass calcd. for $[C_{38}H_{49}N_4O_4]^+$: 625.3748. Measured: 625.3747 (EI+).

***** one H missing due to overlap

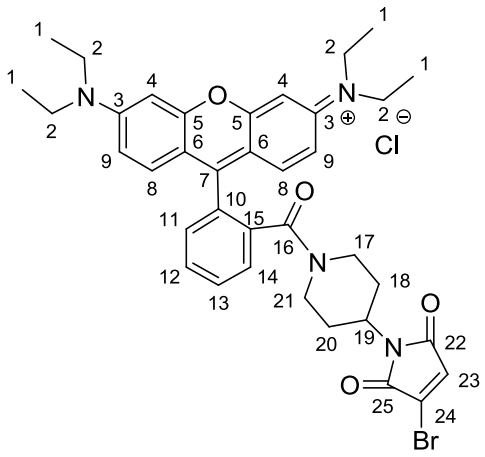
149. 1-(2-(6-(diethylamino)-3-(diethyliminio)-3H-Xanthen-9-yl)benzoyl)piperidin-4-aminium 2,2,2-trifluoroacetate chloride[149]



To a stirred solution of carbamate **148** (230 mg, 0.37 mmol) in CH_2Cl_2 (26 mL) was added TFA (26 mL) and the resulting reaction mixture was stirred at room temperature for 15 h. Toluene (2 x 2 mL) was added to aid evaporation of the solvent to give 240 mg (0.37 mmol, quantitative) of the title compound **149** as a purple solid.

δ_H (600 MHz, MeOD) 7.79-7.75 (2H, m, C*H*-11 and C*H*-14), 7.67-7.65 (1H, dd, *J* = 5.6, 2.2 Hz, C*H*-12 or 13), 7.50 (1H, dd, *J* = 5.8, 2.2 Hz, C*H*-13 or 12), 7.26 (2H, d, *J* = 9.4 Hz, 2 x C*H*-8), 7.08 (2H, t, *J* = 11.2 Hz, 2 x C*H*-9), 6.97 (2H, d, *J* = 1.6 Hz, 2 x C*H*-4), 4.30 (1H, d, *J* = 12.8 Hz, C*H*H), 3.84 (1H, d, *J* = 13.4 Hz, CH*H*), 3.72-3.65 (8H, m, 4 x CH_2-2), 3.31-3.27 (1H, m, C*H*-19), 3.02-2.92 (1H, br s,C*H*H), 2.62-2.52 (1H, br s, CH*H*), 2.00-1.92 (2H, m, CH_2), 1.41-1.29 (14H, m, 4 x CH_3-1 and CH_2) δ_C (150 MHz, $CDCl_3$) 169.6 (2 x C), 159.3 (C), 157.3 (C), 157.2 (C), 156.7 (C), 136.6 (2 x C), 133.4 (CH), 132.9 (CH), 131.9 (CH), 131.8 (CH), 131.4 (CH), 129.3 (CH), 128.5 (CH), 126.3 (CH), 115.4 (CH), 115.3 (CH), 114.9 (C), 114.5 (C), 97.4 (CH), 97.3 (CH), 47.1 (CH_2), 46.9 (4 x CH_2), 41.1 (CH_2), 31.3 (CH_2), 30.5 (CH_2), 12.8 (4 x CH_3); IR 2983 (br), 1677 (s), 1590 (s), 1340 (m), 1199 (s); MS (FAB+) *m/z,* (relative intensity): 525 ($[M]^+$, 100), 497.29 (10). Exact mass calcd. for $[C_{33}H_{41}N_4O_2]^+$: 525.3224. Measured: 525.3211 (NSI+).

150. *N*-(9-(2-(4-(3-Bromo-pyrrole-2,5-dione-1-yl)piperidine-1-carbonyl)phenyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium chloride[149]

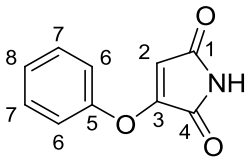


Bromomaleic anhydride (19 µL, 0.20 mmol) was added to rhodamine trifluoroacetate salt **149** (77 mg, 0.15 mmol) in AcOH (4.5 mL). The resulting reaction mixture was heated to 120 °C for 5 h. After this time, toluene (2 x 2 mL) was added to aid the evaporation of the solvent. Purification by flash chromatography (CH_2Cl_2 : MeOH, gradient elution from 98 : 2 to 80 : 20) afforded the title compound **150** as a purple solid (36.6 mg, 0.05 mmol) in 35% yield.

δ_H (600 MHz, MeOD) 7.77-7.74 (2H, m, C*H*-1 and C*H*-4), 7.65-7.64 (1H, m), 7.52-7.50 (1H, m, C*H*-12 or C*H*-13), 7.28-7.26 (2H, m, 2 x C*H*-8), 7.07-7.05 (3H, m, C*H*-24 and 2 x C*H*-9), 7.00-6.98 (2H, m, 2 x C*H*-4), 4.36 (1H, d, *J* = 13.2 Hz, C*H*H), 4.11-4.07 (1H, m, CH), 3.86 (1H, d, *J* = 13.3 Hz, CH*H*), 3.75-3.65 (8H, m, 4 x CH_2-2), 3.19-3.10 (1H, m, C*H*H), 2.63-2.58 (1H, m, CH*H*), 1.80-1.64 (2H, m, CH_2), 1.59-1.51 (2H, m, CH_2), 1.33-1.31 (12H, m, 4 x CH_3-1); δ_C (150 MHz, MeOD)* 169.5 (C), 169.3 (C), 166.3 (C), 159.3 (C), 157.3 (C), 157.2 (C), 156.9 (C), 136.9 (C), 133.4 (CH), 132.2 (C), 132.1 (C), 131.6 (CH), 131.2 (CH), 131.0 (CH), 128.8 (CH), 128.3 (CH), 114.8 (C), 97.7 (CH), 97.2 (CH), 47.0 (CH_2), 46.9 (4 x CH_2), 42.5 (CH_2), 29.5 (CH_2), 12.8 (4 x CH_3); IR 3409 (br), 1713 (m), 1588 (s), 1413 (m), 1338 (m); MS (ES+) *m/z,* (relative intensity): 685 ($[^{81}M]^+$, 87), 683 ($[^{79}M]^+$, 85). Exact mass calcd. for $[C_{37}H_{40}{}^{81}BrN_4O_4]^+$: 685.2202. Measured: 685.2207 (NSI+).

* one CH_2 missing due to overlap.

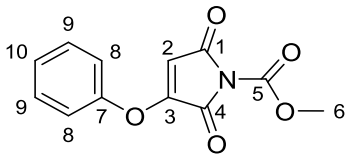
152. 3-Phenoxy-pyrrole-2,5-dione



To melted phenol (3.23 g, 34.3 mmol) a solution of potassium *tert*-butoxide (307 mg, 2.74 mmol) in dry dioxane (2 mL) was added dropwise and the resulting mixture was stirred for 10 min at 40 °C. Then a solution of bromomaleimide **22** (400 mg, 2.28 mmol) in dry dioxane (2 mL) was added dropwise and the resulting mixture was stirred at 40 °C for 30 min. After this time, the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 70 : 30) afforded the title compound **152** as a white solid (328 mg, 1.73 mmol) in a 76% yield.

δ_H (600 MHz, $CDCl_3$) 8.44 (1H, br s, N*H*), 7.43-7.40 (2H, m, 2 x C*H*-Ar), 7.29-7.26 (1H, m, C*H*-8), 7.17-7.15 (2H, m, 2 x C*H*-Ar), 5.28 (1H, s, C*H*-2); δ_C (150 MHz, $CDCl_3$) 170.4 (C=O), 166.1 (C=O), 159.6 (C), 153.9 (C), 130.5 (2 x CH), 127.0 (CH), 119.9 (2 x CH), 100.5 (CH); IR 3264 (br), 1732 (s), 1708 (s), 1627 (s), 1584 (s), 1488 (s), 1288 (s), 1215 (s); MS (EI+) *m/z,* (relative intensity): 189 ($[M]^+$, 85), 94 (100), 84 (95). Exact mass calcd. for $[C_{10}H_7NO_3]^+$: 189.0426. Measured: 189.0420 (EI+); m.p.: 85 °C.

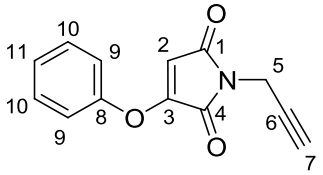
151. Methyl 3-phenoxy-pyrrole-2,5-dione carboxylate



To a solution of phenoxymaleimide **152** (360 mg, 1.41 mmol) in CH_2Cl_2 (6 mL) were added a solution of methyl chloroformate (1.09 mL, 14.1 mmol) in CH_2Cl_2 (1 mL) and a solution of *N*-methylmorpholine (186 µL, 1.69 mmol) in CH_2Cl_2 (1 mL). The reaction mixture was stirred at room temperature for 30 min. After this time, the solution was washed with water (3 x 5 mL), dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo* to afford the title compound **151** as a pale pink solid (329 mg, 1.33 mmol) in 95% yield.

δ_H (600 MHz, $CDCl_3$) 7.46-7.43 (2H, m, 2 x C*H*-Ar), 7.33-7.30 (1H, m, C*H*-10), 7.17-7.15 (2H, m, 2 x C*H*-Ar), 5.42 (1H, s, C*H*-2), 3.98 (3H, s, CH_3-6); δ_C (150 MHz, $CDCl_3$) 164.6 (C=O), 161.0 (C=O), 159.3 (C=O), 153.6 (C), 148.0 (C), 130.6 (2 x CH), 127.4 (CH), 119.8 (2 x CH), 101.6 (CH), 54.4 (CH_3); IR 1769 (s), 1720 (s), 1641 (m), 1304 (s), 1259 (s), 1104 (m), 1075 (m); MS (CI+) *m/z,* (relative intensity): 248 ($[M+H]^+$, 100), 216 (75). Exact mass calcd. for $[C_{12}H_9NO_5]^+$+H: 248.0559. Measured: 248.0554. (CI+); m.p.: 70 - 72 °C.

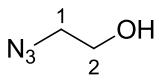
153. 3-Phenoxy-1-(prop-2-ynyl)-pyrrole-2,5-dione



A solution of propargylamine (32.4 µL, 0.50 mmol) in CH_2Cl_2 (0.5 mL) was added to a solution of *N*-methoxycarbonyl phenoxymaleimide **151** (125 mg, 0.50 mmol) in CH_2Cl_2 (5 mL) and left to stir at room temperature for 1.5 h. After this time, the solvent was evaporated *in vacuo.* Purification by flash chromatography (PE : EtOAc, gradient elution from 50 : 50 to 30 : 70) afforded the title compound **153** as a white solid (79.2 mg, 0.35 mmol) in 70% yield.

δ_H (600 MHz, $CDCl_3$) 7.47-7.44 (2H, m, 2 x C*H*-Ar), 7.34-7.31 (1H, m, C*H*-11), 7.20-7.18 (2H, m, 2 x C*H*-Ar), 5.33 (1H, s, C*H*-2), 4.32 (2H, s, CH_2-5), 2.24 (1H, s, C*H*-7); δ_C (150 MHz, $CDCl_3$) 168.5 (C=O), 164.4 (C=O), 159.8 (C), 153.8 (C), 130.5 (2 x CH), 127.1 (CH), 119.9 (2 x CH), 99.8 (CH), 71.8 (CH), 26.9 (CH_2); IR 1715 (s), 1631 (s), 1585 (m), 1310 (s), 1220 (m); MS (CI+) *m/z,* (relative intensity): 228 ($[M+H]^+$, 100). Exact mass calcd. for $[C_{13}H_9NO_3]^+$+H: 228.0661. Measured: 228.0657 (CI+); m.p.: 55 °C.

155. 2-Azidoethanol[200]

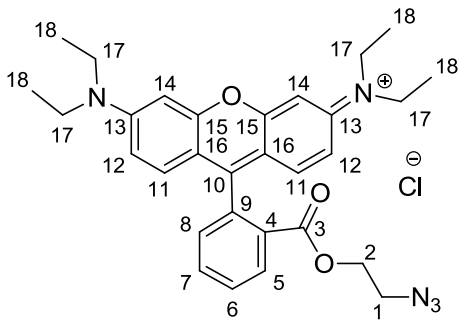


To a round-bottom flask containing sodium azide (2.42 g, 37.2 mmol) and tetrabutylammonium bromide (399 mg, 0.1 mmol) was added 2-chloroethanol (1.00 g, 124 mmol) and the resulting mixture was stirred at 110 °C for 18 h. Purification by

flash chromatography (PE : EtOAc, gradient elution from 70 : 30 to 50 : 50) afforded the title compound as a transparent oil (482 mg, 5.53 mmol) in 45% yield.

δ_H (600 MHz, $CDCl_3$) 3.78 (2H, t, *J* = 6.0 Hz, C*H*$_2$-2), 3.45 (2H, t, *J* = 6.0 Hz, C*H*$_2$-1), 1.80 (1H, br s, O*H*); δ_C (150 MHz, $CDCl_3$) 61.6 (CH_2), 53.6 (CH_2); IR 3350 (br), 2937 (br), 2094 (s), 1288 (m); MS (CI+) *m/z,* (relative intensity): 116 ($[M+K]^+$, 53), 88 ($[M+H]^+$, 100). Exact mass calcd. for $[C_2H_5N_3O]^+$+H: 88.0511. Measured: 88.0513 (CI+).

154. *N*-(9-(2-((2-Azidoethoxy)carbonyl)phenyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium chloride[199]

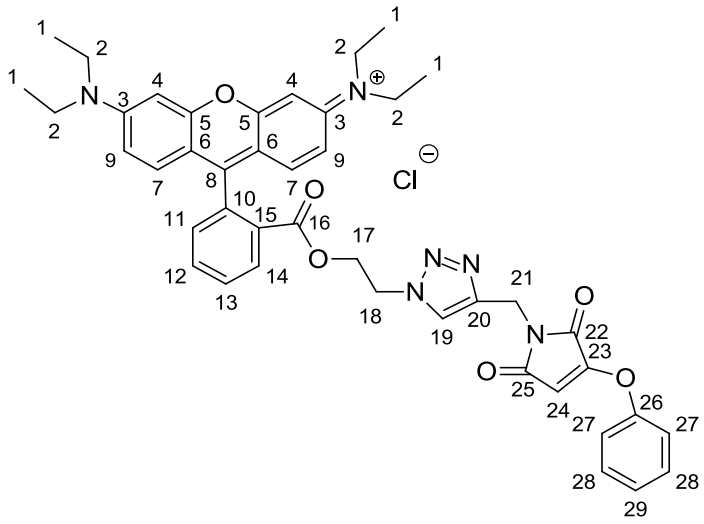


A round bottom flask containing CH_2C_{l2} (1.5 mL) was wrapped in aluminium foil and rhodamine B (100 mg, 0.21 mmol), EDC·HCl (44.1 mg, 0.23 mmol), azide **155** (20 mg, 0.23 mmol) and DMAP (5.30 mg, 0.04 mmol) were added. The resulting reaction mixture was stirred at room temperature for 4 h. After this time, CH_2Cl_2 (5 mL) was added and the reaction mixture was washed with water (10 mL). The aqueous layer was extracted with CH_2Cl_2 (4 x 5 mL) and the organic layers were combined, washed with 0.1 M aq. HCl (5 mL), brine (5 mL), dried (Na_2SO_4), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography ($CHCl_3$: MeOH, 98 : 2) afforded the title compound **154** as a purple solid (115 mg, 0.21 mmol) in 100% yield.

δ_H (600 MHz, $CDCl_3$) 8.24 (1H, d, *J* = 7.8 Hz, C*H*-8 or C*H*-5), 7.77 (1H, m, C*H*-6 or 7), 7.69 (1H, t, *J* = 7.8 Hz, C*H*-6 or C*H*-7), 7.25 (1H, d, *J* = 10.2 Hz, C*H*-8 or C*H*-5), 7.01 (2H, d, *J* = 9.6 Hz, 2 x C*H*-11), 6.85 (2H, dd, *J* = 9.6,2.4 Hz, 2 x C*H*-12), 6.73 (2H, d, *J* = 1.8 Hz, 2 x C*H*-14), 4.11 (2H, t, *J* = 4.8 Hz, C*H*$_2$-2), 3.59-3.56 (8H, m, 4 x C*H*$_2$-17), 3.32 (2H, t, *J* = 4.8 Hz, C*H*$_2$-1), 1.25 (12H, m, 4 x C*H*$_3$-18); δ_C (150 MHz, $CDCl_3$) 164.8 (C), 158.4 (C), 157.8 (2 x C), 155.6 (2 x C), 133.7 (C), 133.5 (CH),

131.6 (CH), 131.3 (2 x CH), 130.6 (CH), 130.4 (CH), 129.4 (C), 114.4 (2 x CH), 113.5 (2 x C), 96.3 (2 x CH), 63.9 (CH_2), 49.6 (CH_2), 46.2 (4 x CH_2), 12.7 (4 x CH_3); IR 3384 (bs), 2971 (w), 2928 (w), 2102 (w), 1721 (m), 1586 (s), 1482 (m), 1413 (m), 1413 (s), 1338 (s), 1180 (s). Exact mass calcd. for $[C_{30}H_{34}N_5O_3]^+$: 512.2656. Measured: 512.2656 (NSI+).

156. *N*-(6-(Diethylamino)-9-(2-((2-(4-((2,5-dioxo-3-phenoxy-2,5-dihydro-1H-pyrrole-1-yl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-*N*-ethylethanaminiumchloride

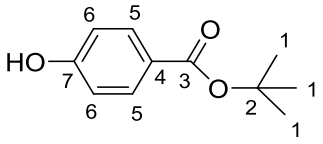


To a mixture of copper iodide (0.19 mg, 1.00 µmol), DIPEA (9.58 µL, 1.00 µmol) and AcOH (9.00 µL, 1.00 µmol) in CH_2Cl_2 (1 mL) was added a mixture of azide **154** (15.5 mg, 0.03 mmol) and alkyne **153** (7.06 mg, 0.03 mmol) in CH_2Cl_2 (0.5 mL). The resulting reaction mixture was stirred at room temperature for 4 h and then the solvent was removed *in vacuo*. Purification by flash chromatography ($CHCl_3$: MeOH, gradient elution 98 : 2 to 90 : 10) afforded the title compound **156** as a purple solid (13.3 mg, 0.02 mmol) in 62% yield.

δ_H (600 MHz, $CDCl_3$) 8.21-8.20 (1H, m, C*H*-11 or C*H*-14), 7.89 (1H, s, C*H*-19), 7.77 (2H, m, C*H*-12 and C*H*-13), 7.46-7.43 (2H, m, 2 x C*H*-Ar), 7.32-7.30 (1H, m, C*H*-Ar), 7.28-7.27 (1H, m, C*H*-11 or C*H*-14) 7.18 (2H, d, *J* = 7.9 Hz, 2 x C*H*-Ar), 7.07 (2H, d, *J* = 9.4 Hz, 2 x C*H*-7), 6.93 (2H, dd, *J* = 9.4, 2.2 Hz, 2 x C*H*-9), 6.79 (2H, d, *J* = 2.3 Hz, 2 x C*H*-4), 5.29 (1H, s, C*H*-24), 4.83 (2H, s, CH_2-21), 4.65 (2H, t, *J* = 5.2 Hz, CH_2-17), 4.49 (2H, t, *J* = 5.2 Hz, CH_2-18), 3.67-3.59 (8H, m, 4 x CH_2-2), 1.32 (12H, t, *J* = 7.1 Hz, 4 x CH_3-1); δ_H (150 MHz,$CDCl_3$) 169.2 (C), 165.1 (C), 164.5 (C), 159.6

(C), 158.7 (C), 157.8 (2 x C), 155.7 (2 x C), 153.9 (C), 142.7 (C), 133.8 (C), 133.4 (CH), 131.8 (CH), 131.5 (2 x CH), 130.9 (CH), 130.5 (2 x CH), 130.3 (CH), 129.3 (C), 127.0 (CH), 123.9 (CH), 119.9 (2 x CH), 114.6 (2 x CH), 113.6 (2 x C), 99.7 (CH), 96.4 (2 x CH), 63.6 (CH_2), 49.0 (CH_2), 46.3 (4 x CH_2), 32.9 (CH_2), 12.8 (4 x CH_3); IR 2970 (w), 1721 (s), 1588 (s), 1411 (m), 1342 (m), 1268 (s), 1247 (s); MS (ES+) *m/z,* (relative intensity): 739 ($[M]^+$, 100), 711 (50). Exact mass calcd. for $[C_{43}H_{43}N_6O_6]^+$:739.3239. Measured: 739.3221 (NSI+).

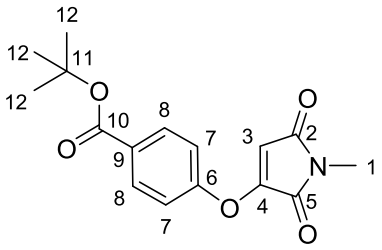
159. *tert*-Butyl 4-hydroxybenzoate[203]



To a solution of 4-hydroxybenzoic acid (2.50 g, 18.1 mmol), DMAP (0.08 g, 0.07 mmol) and *tert*-butanol (50 ml) in dry THF (75 ml), a solution of DCC (3.82 g, 18.5 mmol) in dry THF (25 ml) was added dropwise at room temperature over 30 min. The reaction mixture was stirred at room temperature for a further 22 h. The white solid was filtered and the filtrate was concentrated *in vacuo*. The crude product was dissolved in Et_2O (40 mL), washed with 0.3 M aq. Na_2CO_3 (3 x 20 mL), dried ($MgSO_4$), filtered and then the solvent was evaporated *in vacuo*. Purification by flash chromatography on silica gel (PE : EtOAc, 90 : 10) afforded 1.1 g (5.67 mmol, 31%) of the title compound **159** as a white solid.

δ_H (600 MHz, $CDCl_3$) 7.90 (2H, d, *J* = 8.4 Hz, 2 x C*H*-Ar), 6.83 (2H, d, *J* = 8.4 Hz, 2 x C*H*-Ar), 5.40 (1H, s, O*H*), 1.58 (9H, s, 3 x CH_3-1); δ_C (150 MHz, $CDCl_3$) 165.7 (C=O), 159.4 (C), 131.8 (2 x CH), 124.8 (C), 115.1 (2 x CH), 81.8 (C), 28.4 (3 x CH_3); IR 3338 (br), 1674 (s), 1607 (s), 1368 (s), 1154 (s); IR 3338 (br), 1673 (s), 1607 (s), 1368 (s), 1154 (s); MS (CI+) *m/z,* (relative intensity): 195 ($[M+H]^+$, 100). Exact mass calcd. for $[C_{11}H_{14}O_3]^+$+H: 195.1016. Measured: 195.1013 (CI+); m.p.: 119 °C (lit. m.p.[203]: 118 - 120 °C).

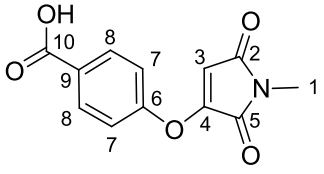
158. *tert*-Butyl 4-(1-methyl-pyrrole-2,5-dione-3-yloxy)benzoate



A solution of *tert*-butyl 4-hydroxybenzoate (300 mg, 1.55 mmol) in dry dioxane (1.5 mL) was added dropwise to a solution of potassium *tert*-butoxide (173 mg, 1.55 mol) in dry dioxane (4.5 mL) and stirred at room temperature for 15 min. The mixture was added dropwise to a solution of bromomaleimide **57** (255 mg, 1.29 mmol) in dry dioxane (1 mL) and the resulting solution was stirred at room temperature for 24 h. After this time, the solvent was concentrated *in vacuo* and the resulting crude redissolved in EtOAc (20 mL), washed with 1 M aq. NaOH (20 mL), water (20 mL) and brine (20 mL). The organic layer was dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo*. Purification by flash chromatography (CH_2Cl_2 : Et_2O, 95 : 5) afforded the title compound **158** as a white solid (260 mg, 0.86 mmol) in 55% yield.

δ_H (600 MHz, $CDCl_3$) 8.05-8.04 (2H, m, 2 x C*H*-Ar), 7.21-7.19 (2H, d, 2 x C*H*-Ar), 5.32 (1H, s, C*H*-3), 3.01 (3H, s, CH_3-1) 1.56 (9H, s, 3 x CH_3-12); δ_C (150 MHz, $CDCl_3$) 169.7 (C=O), 169.7 (C=O), 164.5 (C=O), 158.6 (C), 156.8 (C), 131.9 (2 x CH), 130.7 (C), 119.7 (2 x CH), 100.1 (CH), 81.8 (C), 28.2 (3 x CH_3), 23.8 (CH_3); IR 1712 (s), 1638 (m), 1291 (m); MS (CI+) *m/z,* (relative intensity): 304 ($[M+H]^+$, 30), 248 (100). Exact mass calcd. for $[C_{16}H_{17}NO_5]^+$+H: 304.1179. Measured: 304.1174 (CI+); m.p.: 139 °C.

102. 4-(1-Methyl-pyrrole-2,5-dione-3-yloxy)benzoic acid

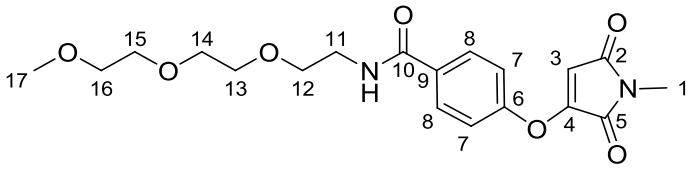


A solution of phenoxymaleimide **158** (250 mg, 0.82 mmol) and thioanisole (1.76 mL, 15 mmol) in CH_2Cl_2 (2 mL) was cooled to 0 °C and added dropwise over 10 min to

TFA (2 mL, 26.3 mmol). The reaction mixture was stirred at room temperature for 4 h and then toluene (2 x 2 mL) was added to aid evaporation of TFA. Purification by flash chromatography (CH_2Cl_2 : MeOH, gradient elution from 95 : 5 to 80 : 20) afforded the title compound **102** as a white solid (123 mg, 0.50 mmol) in 61% yield.

δ_H (600 MHz, MeOD) 8.15 (2H, d, *J* = 9.0 Hz, 2 x C*H*-Ar), 7.38 (2H, d, *J* = 9.0 Hz, 2 x C*H*-Ar), 5.32 (1H, s, C*H*-3), 2.99 (3H, s, CH_3-1); δ_C (150 MHz, MeOD) 171.4 (C=O), 168.7 (C=O), 166.9 (C=O), 159.9 (C), 158.8 (C), 133.2 (2 x CH), 130.6 (C), 120.7 (2 x CH), 101.5 (CH), 23.6 (CH_3); IR 1726 (s), 1678 (m), 1643 (m), 1603 (m), 1294 (m); MS (CI+) *m/z,* (relative intensity): 248 ($[M+H]^+$, 100). Exact mass calcd. for $[C_{12}H_9NO_5]^+$+H: 248.0553. Measured: 248.0550 (CI+); m.p.: 237 °C.

157. *N*-(2-(2-(2-Methoxyethoxy)ethoxy)ethyl)-4-(1-methyl-pyrrole-2,5-dione-3-yloxy)benzamide

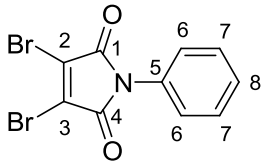


To a solution of phenoxymaleimide **102** (50.0 mg, 0.20 mmol), HOBt (2.93 mg, 0.02 mmol) and HBTU (75.8 mg, 0.20 mmol) in dry DMF (14 mL), DIPEA (34 µL, 0.20 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 20 min. Then a solution of PEG amine **140** (32.2 mg, 0.20 mmol) in dry DMF (2 mL) was added dropwise and the stirring was continued for a further 4 h. After this time, the solvent was evaporated in *vacuo* and the resulting crude was dissolved in CH_2Cl_2 (25 mL), washed with sat. aq. LiCl (2 x 20 mL), 15 % aq. citric acid (10 mL), water (10 mL) and brine (10 mL). The combined organic layers were dried ($MgSO_4$), filtered and then the solvent evaporated *in vacuo*. Purification by flash chromatography (EtOAc : MeOH, gradient elution from 100 : 0 to 80 : 20) afforded the title compound **157** as a yellow oil (52.6 mg, 0.13 mmol) in 67% yield.

δ_H (600 MHz,$CDCl_3$) 7.94-7.92 (2H, m, 2 x C*H*-Ar), 7.25-7.22 (2H, m, 2 x C*H*-Ar), 5.33 (1H, s, C*H*-3), 3.66-3.62 (10H, m, 5 x CH_2), 3.53-3.51 (2H, m, CH_2), 3.31 (3H, s, CH_3-17), 3.05-3.03 (3H, s, CH_3-1); δ_C (150 MHz, $CDCl_3$) 169.7 (C=O), 166.4 (C=O), 165.5 (C=O), 158.8 (C), 155.9 (C), 133.2 (C), 129.8 (2 x CH), 119.9 (2 x CH), 100.1 (CH), 71.8 (CH_2), 70.5 (CH_2), 70.4 (CH_2), 70.2 (CH_2), 70.0 (CH_2), 59.1

(CH_3), 40.0 (CH_2), 23.8 (CH_3); IR 1716 (s), 1639 (m), 1311 (w); MS (CI+) *m/z,* (relative intensity): 393 ($[M+H]^+$, 82), 273 (100). Exact mass calcd. for $[C_{19}H_{24}N_2O_7]^+$+H: 393.1656. Measured: 393.1661 (CI+).

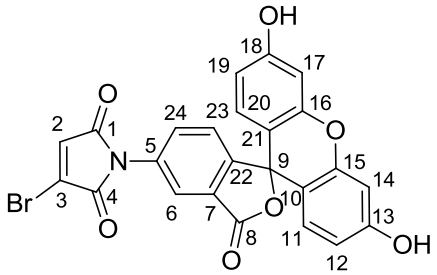
176. 3,4-Dibromo-1-phenyl-pyrrole-2,5-dione[189]



To a solution of dibromomaleic anhydride (300 mg, 1.17 mmol) in AcOH (3.5 mL) was added aniline (118 µL, 1.29 mmol). The reaction mixture was refluxed for 3 h, allowed to cool to room temperature over 15 h and then the solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, gradient elution from 90 : 10 to 80 : 20) afforded the title compound **176** as a yellow solid (91.5 mg, 0.28 mmol) in 24% yield.

δ_H (600 MHz, $CDCl_3$) 7.50-7.47 (2H, m, 2 x C*H*-Ar), 7.43-7.40 (1H, m, C*H*-8), 7.34-7.33 (2H, m, 2 x C*H*-Ar); δ_C (150 MHz, $CDCl_3$) 163.0 (2 x C=O), 131.0 (C), 130.0 (2 x C), 129.5 (2 x CH), 128.8 (CH), 126.2 (2 x CH); IR 1727 (s), 1717 (s), 1369 (w), 1229 (w); MS (CI+) *m/z,* (relative intensity): 334 ($[^{81,81}M+H]^+$, 45), 332 ($[^{79,81}M+H]^+$, 100), 330 ($[^{79,79}M+H]^+$, 45). Exact mass calcd. for $[C_{10}H_5{}^{79,79}Br_2NO_2]^+$+H: 329.9765. Measured: 329.8749 (CI+); m.p.: 169 °C.

179. 3-Bromo-1-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-yl)-pyrrole-2,5-dione[228]

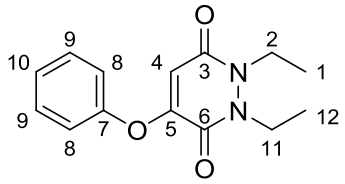


To a solution of fluorescein amine isomer 1 (104 mg, 0.3 mmol) in AcOH (10 mL) was added bromomaleic anhydride (28.7 µL, 0.3 mmol) and the resulting reaction mixture was stirred for 6 h at room temperature, then refluxed for 3 h. After this time

the reaction mixture was allowed to cool to room temperature and the orange precipitate was filtered and dried *in vacuo* to afford the title compound **179** as an orange solid (96.8 mg, 0.19 mmol) in 64% yield.

δ_H (600 MHz, DMSO-d_6) 7.99 (1H, d, *J* = 1.3 Hz, C*H*-6), 7.77 (1H, dd, *J* = 8.3 and 1.9 Hz, CH-24), 7.73 (1H, s, C*H*-2), 7.43 (1H, dd, *J* = 8.1 Hz, C*H*-23), 6.69 (2H, d, *J* = 2.3 Hz, C*H*-14 and C*H*-17), 6.62-6.57 (4H, m, C*H*-19, C*H*-23, C*H*-11 and C*H*-12); δ_C (150 MHz, DMSO-d_6) 167.9 (C=O), 167.6 (C=O), 164.5 (C=O), 159.6 (2 x C), 151.8 (2 x C), 151.5 (C), 133.7 (CH), 133.0 (C), 132.9 (CH), 131.2 (C), 129.1 (2 x CH), 126.7 (C), 124.8 (CH), 122.3 (CH), 112.8 (2 x CH), 109.1 (2 x C), 102.3 (2 x CH), 83.4 (C); IR 3063 (br), 1724 (s), 1579 (s), 1536 (s), 1369 (s), 1208 (s); MS (ES-) *m/z* (relative intensity): 506 ($[^{81}M\text{-}H^+]^-$, 100), 504 ($[^{79}M\text{-}H^+]^-$, 100). Exact mass calcd. for $[C_{24}H_{12}{}^{79}BrNO_7]^+$+H: 505.9875. Measured: 505.9897 (ES+).

197. 1,2-Diethyl-4-phenoxy-1,2-dihydropyridazine-3,6-dione



To a solution of phenol (22.6 mg, 0.24 mmol) in dry dioxane (0.5 mL) a solution of potassium *tert*-butoxide (27.2 mg, 0.24 mmol) in dry dioxane (2 mL) was added dropwise and the solution was stirred for 15 min at room temperature. The resulting mixture was added dropwise to a solution of bromopyridazinedione **187** (50 mg, 0.20 mmol) in dry dioxane (0.5 mL) and the stirring was continued at room temperature for another 18 h. After this time, the solvent was evaporated *in vacuo*. Water (5 mL) was added and the crude product was extracted with EtOAc (3 x 20 mL), washed with brine (5 mL), dried ($MgSO_4$), filtered and the organic solvent was evaporated *in vacuo*. Purification by flash chromatography (PE : EtOAc, 50 : 50) afforded the title compound **197** as a white solid (30.0 mg, 0.11 mmol) in 58% yield.

δ_H (600 MHz, $CDCl_3$) 7.43-7.41 (2H, m, 2 x C*H*-Ar), 7.29-7.26 (1H, m, C*H*-9), 7.11-7.09 (2H, m, 2 x C*H*-Ar), 5.80 (1H, s, CH-4), 4.20 (2H, q., *J* = 7.1 Hz, CH_2-2 or 11), 4.08 (2H, q., *J* = 7.0 Hz, CH_2-2 or 11), 1.30 (3H, t, *J* = 7.2 Hz, CH_3-1 or 12), 1.22 (3H, t, *J* = 7.0 Hz, CH_3-1 or 12); δ_C (150 MHz, $CDCl_3$) 159.5 (C=O), 157.3 (C=O),

154.4 (C), 152.7 (C), 130.7 (2 x CH), 126.7 (CH), 121.4 (2 x CH), 108.3 (CH), 40.7 (CH_2), 40.3 (CH_2), 13.2 (2 x CH_3); IR 1638 (s), 1210 (m); MS (CI+) *m/z* (relative intensity): 261 ($[M+H]^+$, 100); Exact mass calcd. for $[C_{14}H_{16}N_2O_3]^+$+H: 261.1234. Measured: 261.1232 (CI+); m.p.: 130 °C.

V.4 Modification of Grb2 SH2 (L111C)[139] (PDB id: 3MXC)

Sequence of Grb2 SH2 (L111C):

MGIEMKPHPWFFGKIPRAKAEEMLSKQRHDGAFLIRESESAPGDFSLSVKFGDVQ HFKVCRDGAGKYFLWVVKFNSLNELVDYHRSTSVSRNQQIFLRDIEQUIVVPQQPT YVQAGSRSHHHHHH. Calculated mass = 14185.

General procedure for the labelling of Grb2 SH2 (L111C) with monosubstituted maleimides

To a solution of Grb2 SH2 (L111C) **26** (100 µL, 70 µM, 100 mM sodium phosphate buffer, 150 mM NaCl, pH 8.0) at 0 °C were added various amounts (1, 10 or 100 eq.) of the relevant maleimide. In cases when 100 eq. of the labelling reagent were used the stock solution was 400x more concentrated (28.2 mM in DMF) than the peptide solution, while in the rest of the cases the stock solution used was 20x more concentrated (1.41 mM in DMF). The mixture was maintained on ice. Aliquots were taken at various times and immediately analysed by LC-MS. The reaction progress was estimated based on the ratio of the MS peak heights corresponding to the native protein **26** and the resulting protein conjugates(s).

Competition experiments between bromomaleimide 22 and the *N*-Methyl-sulfoxymaleimide 66

Experiment 1: To a mixture of bromomaleimide **22** (1 µL, 2 eq., from a 1.41 mM stock solution in DMF) and sulfoxymaleimide **66** (1 µL, 2 eq., from a 1.41 mM stock solution in DMF) was added Grb2 SH2 (L111C) **26** (10 µL, 70 µM, 100 mM sodium phosphate buffer, 150 mM NaCl, pH 8.0) at 0 °C. The mixture was maintained at 0 °C. After 1 min, an aliquot was taken from the reaction mixture and immediately analysed by LC-MS. The ratio between the products was estimated based on the MS peak heights of the two resulting protein conjugates **113** and **114**.

Experiment 2: To a mixture of bromomaleimide **22** (2 µL, 4 eq., from a 1.41 mM stock solution in DMF) and sulfoxymaleimide **66** (2 µL, 4 eq., from a 1.41 mM stock solution of in DMF) was added a solution of Grb2 SH2 (L111C) (10 µL, 70 µM, 100

mM sodium phosphate buffer, 150 mM NaCl, pH 8.0) at 0 °C. The mixture was maintained at 0 °C. After 1 min, an aliquot was taken from the reaction mixture and immediately analysed by LC-MS. The ratio between the products was estimated based on the MS peak heights of the two resulting protein cojugates **113** and **114.**

General procedure for the labelling of Grb2-SH2 (L111C) with disubstituted maleimides

To a solution of Grb2 SH2 (L111C) **26** (70 µM, 100 mM sodium phosphate buffer, 150 mM NaCl, pH 8.0) at 0 °C was added the relevant disubstituted maleimide (1 eq., from a 1.41 mM stock solution in DMF). The mixture was maintained at 0 °C. Aliquots were taken at various times and immediately analysed by LC-MS. The reaction progress was estimated based on the ratio of the MS peak heights corresponding to the native protein **26** and the resulting protein conjugate(s).

V.5 Modification of somatostatin

Sequence of somatostatin:
AGCKNFFWKTFTSC (cyclic, Cys3-Cys14). Calculated mass = 1636.

Reduction of somatostatin

A solution of somatostatin **29*** (200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (1.5 eq., 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was checked as described below.

* Reactions were performed starting from 50 - 700 µL somatostatin solution.

General procedure for checking the completion of the reduction of somatostatin

To an aliquot from the reduced somatostatin solution **118** (30 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5 % DMF) was added 2,3-dibromomaleimide (3 µL, 10 eq., from a 20 mM stock solution in DMF). The reaction mixture was left to stand at room temperature for 1 min. Completion of the reaction was then checked by LC-MS, based on the disappearance of the MS peak corresponding to the native peptide and appearance of the peak corresponding to the resulting peptide bridged conjugate **119**.

General procedure for the stepwise bridging of somatostatin with dibromomaleimides

A solution of somatostatin **29** (200µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (3 µL, 1.5 eq., 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was determined, as described above, by LC-MS. The reduced somatostatin solution was then treated with the relevant 2,3-dibromomaleimides (4 µL, 2 eq., from a 20 mM stock solution in DMF) and the reaction was left to stand at room temperature for 1 h. Completion of the reaction was checked by LC-MS, based on the disappearance of the MS peak corresponding to native peptide **29** and appearance of the peak corresponding to the resulting peptide conjugat(s).

General procedure for the stepwise bridging of somatostatin with disubstituted maleimides

A solution of somatostatin **29** (100 µL, 153 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (1.2 µL, 1.2 eq., from a 15.3 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was determined, as described above, by LC-MS. To the solution of reduced somatostatin were added various amounts (1 - 100 eq.) of the relevant disubstituted maleimide and the reaction mixture was left to stand at room temperature. In cases when 100 eq. of the labelling reagent were used, the stock solution was 1000x more concentrated (153 mM in DMF) than the peptide solution, while in the rest of the cases the stock solution used was 100x more concentrated (15.3 mM stock solution in DMF). Aliquots were taken at various times and immediately analysed by LC-MS. The reaction progress was estimated based on the ratio of the MS peak heights corresponding to the native peptide **29** and the resulting peptide conjugatet(s).

Competition experiment between iodophenoxymaleimide 108 and dibromomaleimide 25

To a mixture of dibromomaleimide **25** (0.5 µL, 1 eq., from a 15.3 mM stock solution in DMF) and iodophenoxymaleimide **108** (0.5 µL, 1 eq., from stock solution 15.3 mM In DMF) was added a solution of reduced somatostatin **118** (50 µL, 153 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) at room temperature. The mixture was maintained at room temperature for 1 min and then an aliquot was

taken and immediately analysed by LC-MS. Completion of the reaction was checked by LC-MS, based on the disappearance of the MS peak corresponding to the reduced peptide **118** and appearance of the peak corresponding to the resulting peptide conjugats **119** and **120**.

General procedure for the *in situ* bridging of somatostatin with disubstituted maleimides

A solution of somatostatin **29*** (153 μM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was mixed with various amounts (1 or 100 eq.) of the relevant disubstituted maleimides. To the resulting mixture was added TCEP (1.5 equiv, from a 20 mM stock solution in the same buffer) and the mixture was left to stand at room temperature. In cases when 100 eq. of the labelling reagent were used the stock solution was 1000x more concentrated (153 mM in DMF) than the peptide solution, while when 1 eq. of the labelling reagent was used the stock solution used was 100x more concentrated (15.3 mM in DMF). Aliquots were taken at various times and immediately analysed by LC-MS. The reaction progress was estimated based on the ratio of the MS peak heights corresponding to the native peptide **29** and the resulting peptide conjugate.

* Reactions were performed starting from 100 - 700 μL somatostatin solution.

Stepwise bridging of somatostatin with phenoxymaleimides 101, 139 and 146

A solution of somatostatin **29*** (200 μM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (1.5 eq., from a 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was assumed, as described above, by LC-MS. The reduced somatostatin solution was then treated with the relevant phenoxymaleimide (1.5 eq., from a stock solution of 20 mM in DMF) and the reaction left to stand at room temperature for 1 h. Completion of the reaction was checked by LC-MS, based on the disappearance of the MS peak corresponding to the reduced peptide **118** and appearance of the peak corresponding to the resulting peptie conjugate.

* Reactions were performed starting from 100 - 700 μL somatostatin solution.

Stepwise bridging of somatostatin with *N*-Rhodamine phenoxymaleimide 156

A solution of somatostatin **29** (100 μL, 200 μM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (1.2 μL, 1.2 eq., from a 20 mM

stock solution in DMF) and then *N*-Rhodamine phenoxymaleimide **156** (1.2 µL, 1.2 eq., from a 20 mM stock solution in DMF) was added. The reaction mixture was left at room temperature for 30 min and after that TCEP (1.2 µL, 0.6 eq., from a 20 mM stock solution in the same buffer) was added. Completion of the reaction after another 30 min of incubation at room temperature was assumed by LC-MS, based on the disappearance of the MS peak corresponding to the native peptide and appearance of the resulting protein conjugate **162**.

Competition experiment between phenoxymaleimide 101 and bromomaleimide 22

A solution of somatostatin **29** (200 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (3 µL, 1.5 eq., from a 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was determined, as described above, by LC-MS. The reduced somatostatin solution was divided into two equal volumes and added to a mixture of *N*-methyl phenoxymaleimide **101** and bromomaleimide **22** (1 : 1 molar ratio, 1.5 eq., from a 20 mM stock solution in DMF) and to a mixture of **101** and **22** (5 : 1 molar ratio, 7.5 eq. **101** and 1.5 eq. **22**, from a 20 mM stock solution in DMF) respectively. The two reaction mixtures were left to stand at room temperature for 5 min and then checked by LC-MS. The reaction progress was monitored by LC-MS over 1 h, based on the heights of the MS peaks corresponding to the resulting peptide conjugates **127** and **130**.

Procedure for the *in situ* bridging of somatostatin with phenoxymaleimides 101 and 146

A solution of somatostatin **29** (200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was mixed with the relevant phenoxymaleimide (1.5 eq., from a 20 mM stock solution in DMF). To the resulting mixture was added TCEP (1.5 equiv, from a 20 mM stock solution in the same buffer) and the reaction mixture was left to stand at room temperature for 2 h. Completion of the reaction was determined by LC-MS, based on the disappearance of the peak corresponding to the native peptide **29** and appearance of the peak corresponding to the resulting peptide conjugate.

Organic solvent free *in situ* bridging of somatostatin with phenoxymaleimide 157

A solution of somatostatin (200 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4) was mixed with phenoxymaleimide **157** (6 µL, 1.5 eq., from a 10 mM stock solution in the same buffer). To the resulting mixture was added TCEP (3 µL, 1.5 equiv, from a 20 mM stock solution in the same buffer) and the reaction mixture was left to stand at room temperature for 4 h. Completion of the reaction was determined by LC-MS, based on the disappearance of the MS peak corresponding to the native peptide **29** and appearance of the peak corresponding to the resulting peptide conjugate **128**.

Reaction of succinimide bridged somatostatin conjugate 128 with maleimide

Succinimide bridged somatostatin conjugate **128** (100 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was treated with maleimide (1 µL, 1 eq., from a 20 mM stock solution in DMF) and the reaction mixture was left to stand at room temperature for 5 min. Then, an aliquot was taken and immediately analysed by LC-MS to indicate that no reaction had occured.

Thiol stability of maleimide and succinimide bridges

Unpurified somatostatin conjugates **120** and **128** (100 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) were treated with 2-mercaptoethanol (10 µL, 100 eq., from a 200 mM stock solution in DMF) or reduced glutathione (10 µL, 100 eq., from a 200 mM stock solution in 50 mM phosphate buffer, pH 6.4). The reaction mixtures were left to stand on ice. Aliquots were taken from the reaction mixture after 1 h and 13 h and the reaction progress was monitored by LC-MS, based on the ratio between the MS peak height corresponding to the peptide conjugates under investigation and the MS peak height corresponding to the reduced peptide **118**.

Thiol stability of succinimide and maleimide bridges under cytoplasm mimicking conditions

Succinimide bridged somatostatin conjugates **120** and **128** were dialysed (Slide-A-Lyzer MINI Dialysis Devices, 2K MWCO), 5000x dilution into 20 mM HEPES buffer (100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4). The concentration of the peptide

conjugates was adjusted to 100 μM and the resulting reaction mixtures* were incubated at 37 °C for 21 h in the presence of 1 mM reduced glutathione (from a 20 mM stock solution in the same buffer). Aliquots from the reaction mixtures were taken at various times and were immediately analysed by LC-MS. The progress of the reactions was estimated, based on the ratio of the peak heights corresponding to the MS peptide conjugates under investigation and the MS peak height corresponding to the reduced peptide **118**.

* Reactions were done starting from 100 and 300 μL of peptide conjugates.

Hydrolytic stability of maleimide and succinimide bridges

Unpurified somatostatin conjugates **128, 174, 175** (200 μM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) were dialysed (Slide-A-Lyzer MINI Dialysis Devices, 2K MWCO), 5000x dilution into pH 8 buffer (50 mM sodium phosphate) for 12 h at 0 °C. The concentration of the peptide conjugates was adjusted to 100 μM and the resulting reaction mixtures (100 μL) were incubated at 37 °C. The progress of the reaction was monitored by LC-MS, based on the ratio of the MS peak heights corresponding to the peptide conjugates under investigation and the MS peak height corresponding to the hydrolysed peptide conjugates.

Thiol stability of hydrolysed succinimide and maleimide bridges

Hydrolysed somatostatin conjugates **174** and **175** (100 μL, 200 μM, 50 mM sodium phosphate, pH 8), were treated with 2-mercaptoethanol (10 μL, 100 eq., from a 200 mM stock solution in DMF). The reaction mixture was incubated at 37 °C for 21 h. The progress of the reaction was monitored by LC-MS to indicate that no reaction had occurred.

General procedure for the dual labelling of somatostatin

Succinimide bridged somatostatin conjugate **128, 161** and **162** (200 μM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was treated with various amounts of the relevant bromomaleimide (from a 20 mM stock solution in DMF), (Table 6) and heated at 37 °C for 2 h. Completion of the reaction was confirmed by LC-MS, based on the disappearance of the peak corresponding to the starting succinimide bridged conjugate and the appearance of the peak of the resulting dual conjugate.

* Reactions were performed starting from 100 or 200 μL of peptide conjugate.

Dual labelled conjugate	Reagent code (eq.)
177	Bromomaleimide **22** (1.5 eq.)
180	*N*-Fluorescein bromomaleimide **179** (4 eq.)
181	Bromomaleimide **57** (1.5 eq.)
182	*N*-Fluorescein bromomaleimide **179** (4 eq.)

Table 6. Amounts of bridging reagents used for the dual labelling of somatostatin

Preparation of the dual labelled somatostatin conjugate with a maleimide and succinimide tag 178

Succinimide bridged somatostatin conjugate **128** (100 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40 % CH_3CN, 2.5% DMF) was treated with maleimide (5 µL, 5 eq., from a 20 mM stock solution in DMF) and the resulting reaction mixture was left to stand at room temperature for 12 h. Completion of the reaction was determined by LC-MS, based on the disappearance of the MS peak corresponding to the succinimide bridged somatostatin conjugate **128** and appearance of the peak corresponding to the resulting dual labelled peptide conjugate **178**.

Thiol cleavage of dual labelled somatostatin conjugate 177

Dual labeled somatostatin conjugate **177 (**100 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40 % CH_3CN, 2.5% DMF), prepared as described above, was treated with 2-mercaptoethanol (10 µL, 100 eq., from a 200 mM stock solution in DMF) and the reaction mixture incubated at 37 °C for 3 h. Complete cleavage of the 2 labels to afford reduced somatostatin was determined by LC-MS, based on the disappearance of the MS peak corresponding to the dual labelled somatostatin **177** and appearance of the peak corresponding to the reduced somatostatin **118**.

Trypsin digest of the dual labelled somatostatin conjugate 181

A solution of dual labelled somatostatin conjugate **181** (200 µM, 50 mM sodium phosphate, pH 6.4, 40 % CH_3CN, 2.5% DMF) was dialysed (Slide-A-Lyzer MINI Dialysis Devices, 2K MWCO), 5000x dilution into pH 8.1 buffer (100 mM ammonium acetate, 1 mM calcium chloride). The concentration of the peptide conjugate was adjusted to 100 µM and to the resulting reaction mixture (100 µL) was added trypsin

(1 µL, 0.1 eq., from a 1 mM stock solution in the same buffer). The reaction mixture was incubated at 37 °C for 15 h and then the reaction mixture was analysed by LC-MS.

Screening of reducing reagents for trapping the intermediate of the retro-Michael addition reaction

DTT

A solution of succinimide bridged somatostatin conjugate* **128** (200 µM, 50 mM sodium phosphate, pH 6.4, 40 % CH_3CN, 2.5% DMF), was mixed with added various amounts (10 - 250 eq.) of DTT (from a 100 mM stock solution in the same buffer). The reaction was incubated at 37 °C for 1.5 or 2 h. After this time, various amounts (100 - 300 eq.) of maleimide (from a 400 mM stock solution in the same buffer) were added to quench the reaction. When 250 eq. of DTT was added, the reaction mixture was dialysed (Slide-A-Lyzer MINI Dialysis Devices, 2K MWCO), 5000x dilution into pH 8 buffer (50 mM sodium phosphate) for 4 h at 0 °C prior to quenching and the concentration was readjusted to 200 µM. The progress of the reactions was monitored by LC-MS, based on the ratio between the MS peak heights of the peptide conjugates **201** and **202**.

* Reactions were done starting from 100 and 300 µL of somatostatin conjugate **128**.

TCEP

A solution of succinimide bridged somatostatin conjugate **128** (200 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF), prepared as described above, was mixed with TCEP (26 µL, 65 eq., from a 100 m stock solution in the same buffer) and the reaction mixture was incubated at room temperature for 6 h. After this time, maleimide (40 µL, 100 eq., from a 100 mM stock solution in the same buffer) was added to quench the reaction. The progress of the reactions was monitored by LC-MS to show that no reaction had occured.

V.6 Modification of octreotide

Sequence of octreotide:

FCFWKWCW (cyclical, C2-C7), acetate salt. Calculated mass: 1019.

Reduction of octreotide

A solution of octreotide **163*** (200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (1.5 eq., 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was checked as described below.

* Reactions were performed starting from 50 - 500 µL octreotide solution.

General procedure for checking the completion of the reduction of octreotide

To an aliquot from the reduced octreotide solution (30 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4) was added 2,3-dibromomaleimide (3 µL, 10 eq., from a 20 mM stock solution in DMF). The reaction mixture was left to stand at room temperature for 1 min. Completion of the reaction was then checked by LC-MS, based on the disappearance of the MS peak corresponding to the native peptide and appearance of the peak corresponding to peptide conjugate.

Stepwise bridging of octreotide with phenoxymaleimide 103

A solution of octreotide **163** (100 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (2 µL, 2 eq., from a 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was confirmed, as described above, by LC-MS. The reduced octreotide solution was then treated with phenoxymaleimide **103** (1.2 µL, 1.2 eq., from a stock solution of 20 mM in DMF) and the reaction left to stand at room temperature for 1 h. Completion of the reaction was determined by LC-MS, based on the disappearance of the MS peak corresponding to the reduced peptide and appearance of the peak corresponding to peptide conjugate **164**.

Stepwise bridging of octreotide with bromopyridazinedione 187

A solution of octreotide **163** (100 µL, 200 µM, 50 mM sodium phosphate buffer, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (2 µL, 2 eq., from a 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the

reduction was determined, as described above, by LC-MS. The reduced octreotide solution was treated various amounts (5 or 10 eq.) of bromopyridazinedione **187** (from a 20 mM stock solution in DMF) and the reaction was left to stand at room temperature for 1 h. The progress of the reaction was checked by LC-MS, based on the MS peak heights corresponding to the native peptide and the resulting peptide conjugates **189** and **190**.

Stepwise bridging of octreotide with phenoxypiridazinedione 197

A solution of octreotide **163** (200 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was reduced with TCEP (4 µL, 2 eq., from a 20 mM stock solution in the same buffer) for 1 h at room temperature. Completion of the reduction was determined, as described above, by LC-MS. The reduced octreotide solution (50 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was then treated with various amounts (5 - 500 eq.) of phenoxypyridazinedione **197** and the reaction was left to stand at 37 °C. In cases when more than 100 eq. of the labelling reagent were used the stock solution was 5000x more concentrated (1 M in DMF) than the peptide solution, while in the rest of the cases the stock solution used was 100x more concentrated (20 mM in DMF). The progress of the reaction was checked by LC-MS, based on the heights of the MS peak corresponding to the reduced peptide and peptide conjugates **189** and **190**.

***In situ* bridging of octreotide with phenoxymaleimides 101 and 146**

A solution of octreotide **163** (100 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was mixed with the relevant phenoxymaleimide (1.5 µL, 1.5 eq., from a 20 mM stock solution in DMF). To the resulting solution was added TCEP (1.5 equiv, from a 20 mM stock solution in the same buffer) and the mixture was left to stand at room temperature for 3 h. The completion of the reaction was determined by LC-MS, based on the disappearance of the MS peak corresponding to the native peptide **163** and appearance of the peak corresponding to the resulting peptide conjugate.

pH screen for the dual labelling of octreotide with bromomaleimide 22

Succinimide bridged octreotide conjugate **164** (600 µL, 200 µM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was divided in 4 equal volumes and dialysed (Slide-A-Lyzer MINI Dialysis Devices, 2K MWCO), 5000x dilution into 4

different buffers: 1) pH 8 buffer (50 mM sodium phosphate); 2) pH 7 (50 mM sodium phosphate); 3) pH 6 (50 mM sodium phosphate); 4) pH 5 (50 mM sodium citrate). The concentration of the dialysed solutions was adjusted to 174.5 μM and then bromomaleimide **22** (5 eq., 20 mM stock solution in DMF) was added. The resulting reaction mixtures were incubated at 37 °C for 18 h. The progress of the reaction was monitored by LC-MS, based on the heights of the MS peaks corresponding to the peptide conjugates **183** and **184**.

Dual labelling of octreotide with maleimide

Succinimide bridged octreotide conjugate **164** (100 μL, 200 μM, 50 mM sodium phosphate, pH 6.4, 40 % CH_3CN, 2.5% DMF) was treated with maleimide (5 μL, 5 eq., from a 20 mM stock solution in DMF) and the reaction mixture was incubated at 37 °C for 2 h. The progress of the reaction was monitored by LC-MS, based on the heights of the MS peaks corresponding to the resulting peptide conjugates.

Dual labelling of octreotide with phenoxymaleimide 101

Succinimide bridged octreotide conjugate **191** (100 μL, 200 μM, 50 mM sodium phosphate, pH 6.4, 40 % CH_3CN, 2.5% DMF) was treated with *N*-methyl phenoxymaleimide **101** (15 μL, 15 eq., from a 20 mM stock solution in DMF) and the reaction mixture was incubated at 37 °C for 30 h. The progress of the reaction was monitored by LC-MS, based on the heights of the MS peaks corresponding to conjugates **192** and **193**.

Dual labelling of octreotide with pyridazinediones 186, 187 and propiolamide 188

Succinimide bridged octreotide conjugate **164** (200 μM, 50 mM sodium phosphate, pH 6.4, 40% CH_3CN, 2.5% DMF) was treated with various amounts (1.5 - 300 eq.) of the relevant labelling reagent and the reaction mixture incubated at 37 ^oC for various periods of time (2 h - 5 days). In cases when more than 100 eq. of the labelling reagent were used the stock solution was 5000x more concentrated (1 M in DMF) than the peptide solution, while in the rest of the cases the stock solution used was 100x more concentrated (20 mM in DMF). The progress of the reactions was monitored by LC-MS, based on the heights of the MS peaks corresponding to the resulting peptide conjugates.

V.7 Modification of an anti-CEA ds-scFv (representation of the protein was taken from Baker et al.)[146]

Sequence of anti-CEA ds-scFv:
AGIHVAQPAMAQVKLEQUIVSGAEVVKPGASVKLSCKASGFNIKDSYMHWLRQGP
QCLEWIGWIDPENGDTEYAPKFQGKATFTTDTSANTAYLGLSSLRPEDTAVYYCN
EGTPTGPYYFDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSENVLTQSPS
SMSASVGDRVTIACSASSSVPYMHWFQQKPGKSPKLLIYSTSNLASGVPSRFSGS
GSGTDYSLTISSVQPEDAATYYCQQRSSYPLTFGCGTKLEIKHHHHHH. Calculated mass = 27531.

In situ bridging of ds-scFv with *N*-methyl phenoxymaleimide 101

To a solution of ds-scFv **33** (50 µL, 70 µM, pH 7.4 PBS) were added phenoxymaleimide **101** (0.25 µL, 5 eq., from a 70 mM stock solution in DMF) and benzeneselenol (2.5 µL, 25 eq., from a 35 mM stock solution in DMF). The reaction mixture was left to stand at room temperature for 30 min. Completion of the reaction was determined by LC-MS, based on the disappearance of the MS peak corresponding to the native protein **33** and appearance of the peak corresponding to the protein conjugate **169**.

In situ bridging of ds-scFv with *N*-PEG-phenoxymaleimide 146

To a solution of ds-scFv **33** (50 µL, 70 µM, pH 7.4 PBS) were added phenoxymaleimide **146** (0.75 µL, 15 eq., from a 70 mM stock solution in DMF) and benzeneselenol (5 µL, 50 eq., from a 35 mM stock solution in DMF). The reaction mixture was left to stand at room temperature for 30 min. Completion of the reaction was confirmed by LC-MS, based on the disappearance of the MS peak corresponding to the native protein **33** and appearance of the peak corresponding to the protein conjugate **170**.

Thiol stability of succinimide bridged ds-scFv conjugate 169

Unpurified ds-scFv conjugate **169** (100 µL, 70 µM, pH 7.4 PBS) was treated with 2-mercaptoethanol (10 µL, 100 eq., 70 mM stock solution in DMF). The same experiment was repeated using reduced glutathione (10 µL, 100 equiv, 70 mM stock solution in pH 7.4 PBS). The reaction mixtures were left to stand at room

temperature for 48 h. The reaction progress was monitored by LC-MS to show that no reaction had occurred.

General procedure for the dual labelling of ds-scFv

Succinimide bridged ds-scFv conjugate **169** (200 µL, 70 µM, pH 7.4 PBS) was purified on a PD MiniTrap G-25 (GE Healthcare) desalting column and concentrated by centrifugation using 10 KDa MWCO filters. The protein concentration was adjusted to 70 µM (pH 7.4 PBS, 10% DMF) and then bromomaleimide **22** (50 eq., from a 70 mM stock solution in DMF) was added. To the resulting solution was added guanidinium chloride (from a 6M stock solution in PBS) to afford a final concentration of 2 M. The reaction mixture was incubated at 37 °C for 2 h. The completion of the reaction was confirmed by LC-MS, based on the disappearance of the MS peak corresponding to the succinimide bridged protein conjugate **169** and appearance of the peak corresponding to the dual labelled proetein conjugate **203**.

V.8 Modification of Rituximab-Fab (PDB id: 2OSL)

Sequence of the light chain:
QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPG
NGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYF
NVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV.
Calculated mass: 23167.
Sequence of the heavy chain:
QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPG
NGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYN
VWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKEK
SC. Calculated mass: 23712.

Stepwise bridging of Rituximab Fab with *N*-PEG phenoxymaleimide 146

A solution of Rituximab Fab **165** (50 µL, 22.9 µM, PBS buffer 7.4) was reduced with DTT (10 µL, 200 eq., from a 22.9 mM stock solution in the same buffer) for 5 h at room temperature. Alternatively, the reduction was achieved by using TCEP (3.4 µL, 30 eq., from a 10 mM stock solution in the same buffer) and incubating the reaction mixture at room temperature for 2 h. After this time, the Fab solution was buffer

swapped in pH 7.4 PBS (1 mM EDTA) using 10 kDa MWCO filters. The concentration of the reduced Fab was adjusted to 20.1 μM. Completion of the reduction was determined by LC-MS, based on the disappearance of the MS peak corresponding to the native Fab. The reduced Fab solution (30 μL) was then treated with *N*-PEG phenoxymaleimide **146** (1.5 μL, 5 eq., from a 2.01 mM stock solution in DMF) and the reaction left to stand at room temperature for 1 h. The completion of the reaction was determined by LC-MS, based on the disappearance of the peaks corresponding to the light and heavy chains and appearance of the peak corresponding to conjugate 166.

V.9 Modification of Herceptin Fab (PDB id: 1N8Z)

Sequence of the light chain:
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN
GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP.
Calculated mass = 23404.
Sequivuence of the heavy chain:
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYS
GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAA
PSVFIFPPSDEQUIVLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QUIVDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC.
Calculated mass = 23443.

Preparation of Herceptin Fab fragment using pepsin and papain digest[212]

Immobilised pepsin (0.15 mL) (Thermo Scientific) was buffer swapped 4x with digestion buffer (20 mM sodium acetate trihydrate, pH 3.1). After this, Herceptin (0.5 mL, 6.41 mg/mL in digestion buffer) was added and the resulting mixture was incubated for 5 h at 37 °C while shaking at 1100 rpm. The digest was separated from the resin using a filter column (Pierce Centrifuge Column 0.8 mL, Thermo Scientific) and the digest was buffer swapped 3x with digest buffer (50 mM sodium phosphate, 1 mM EDTA, 150 mg NaCl, pH 6.8) using 10 kDa MWCO filters (Vivaspin 6 mL, Santorius). The digest was mixed with the washes and the volume adjusted to 0.5 mL. After this, the papain resin (1 mL, 0.25 mg/mL) (Thermo Scientific) was treated with DTT (from a 10 mM stock solution in digest buffer), to

activate it, for 1 h at 25 °C, under argon, in the dark while shaking (1100 rpm). The resin was washed with digest buffer and then the 0.5 mL of antibody solution was added. The mixture was incubated at 37 °C for 16 h, and in the dark, with shaking (1100 rpm). The resin was separated from the digest using a filter column (Pierce Centrifuge Column 0.8 mL, Thermo Scientific), and buffer swapped 3x with PBS (pH 7.4). The digest was mixed with the washes and the buffer was exchanged for PBS (pH 7.4) using 10 kDa MWCO filters (Vivaspin 6 mL, Santorius) to give a final volume of 0.4 mL. The digest was analysed by SDS-PAGE and LCMS to reveal a single Herceptin Fab fragment **167**, observed mass: 47652. The concentration of Herceptin Fab was determined by UV/VIS spectroscopy using a molecular extinction coefficient of ε_{280}= 68590 M^{-1} cm^{-1}. [Herceptin Fab fragment] 2 mg/L (0.4 mL), 38 %.

Sequential bridging of Herceptin Fab with phenoxymaleimide 101

A solution of Herceptin Fab **167** (50 µL, 22.9 µM, pH 8 borate buffer) was reduced with TCEP (1.5 µL, 3 eq., from a 2.29 mM stock solution in the same buffer) and then incubated at 37 °C for 2 h, with shaking (400 rpm). Completion of the reduction was determined by LC-MS, based on the disappearance of the MS peaks corresponding to the unreacted Fab. The reduced Fab solution (30 µL) was then treated with *N*-methyl phenoxymaleimide **101** (0.6 µL, 2 eq., 2.29 mM stock solution in DMF) and the reaction was left to stand at room temperature for 1 h. The completion of the reaction was checked by LC-MS, based on the disappearance of the peaks corresponding to the light and heavy chains.

V.10 Kinetic study

Reactions with 2-mercaptoethanol were carried out at room temperature, while reactions with GSH were carried out at 22 °C.

Experiments with 2-mercaptoethanol

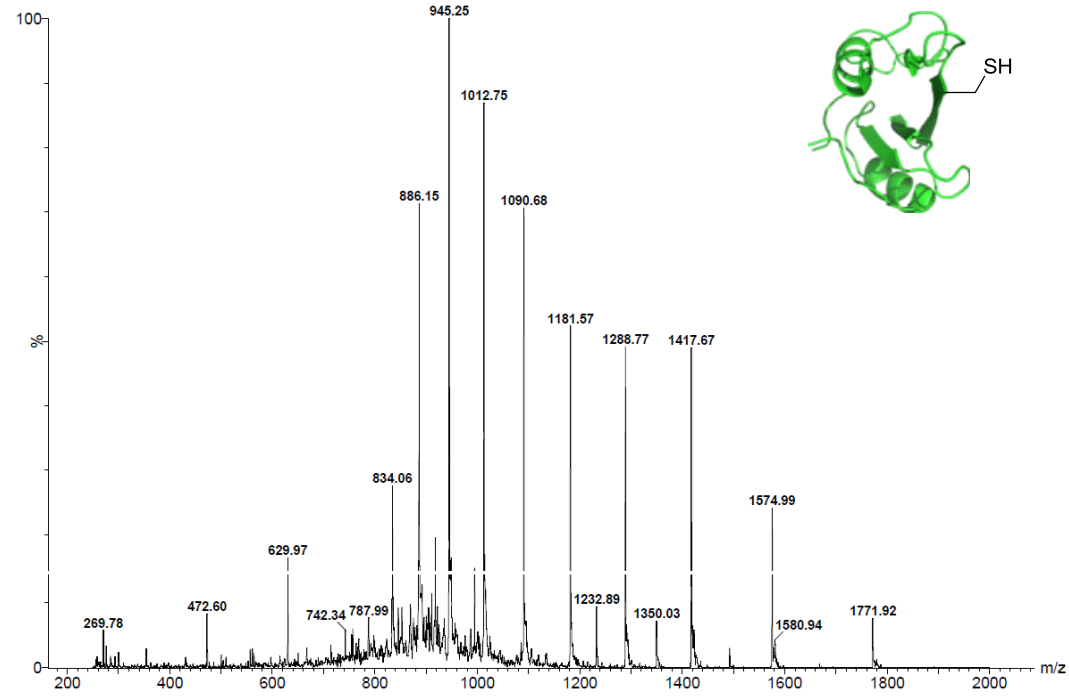
To the cuvette containing the relevant substituted maleimide **57**, **86**, **88** or **101** (50 µL, 500 µM stock solution in pH 6 buffer, 50 mM phosphate, 20% CH_3CN) in the background solution (50 µL, same buffer) was added freshly prepared 2-mercaptoethanol (50 µL, from a 500 µM stock solution in the same buffer) and the acquisition of the signal at 360 nm was started. In the case of halomaleimides **57**, **86**, **88** the acquisition was stopped after 10 min while in the case of phenoxymaleimide **101** the acquisition was stopped after 60 min.

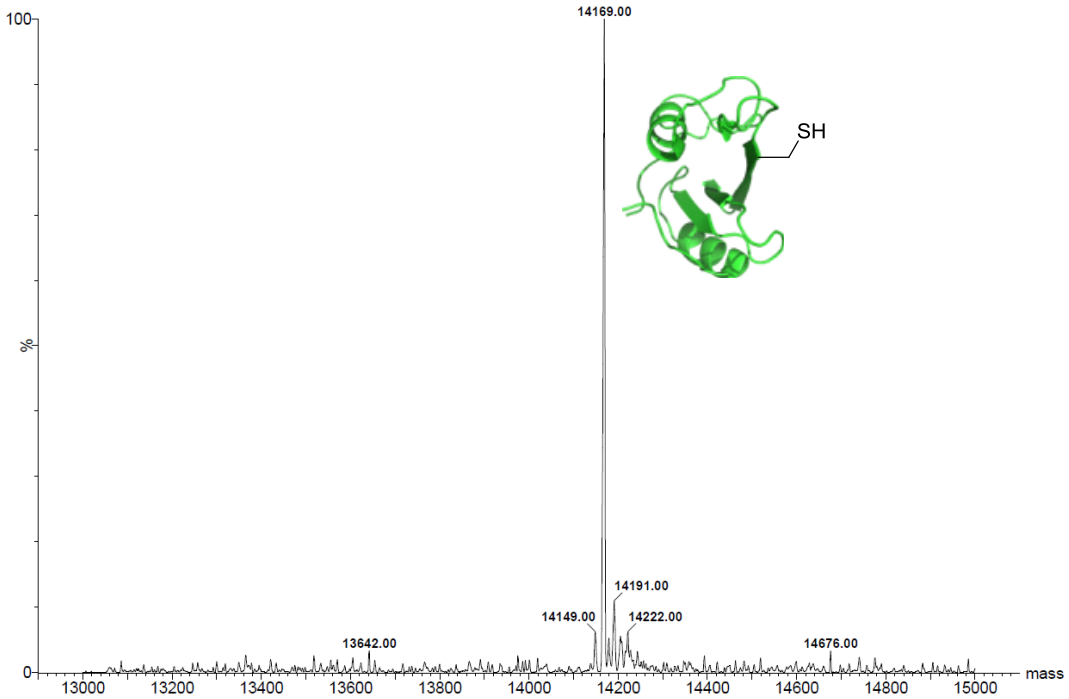
Experiments with glutathione

To the cuvette containing reduced GSH (75 µL, 300 µM stock solution in pH 6 buffer (50 mM phosphate buffer)) was added the relevant monosubstituted maleimide **57**, **86**, **88** or **101** (75 µL, 300 µM stock solution pH 6 buffer, 50 mM sodium phosphate, 20% CH_3CN) and the acquisition of the signal at 360 nm was started. The acquisition was stopped after 30 min. The reaction mixture was also analysed by ^{1}H-NMR.

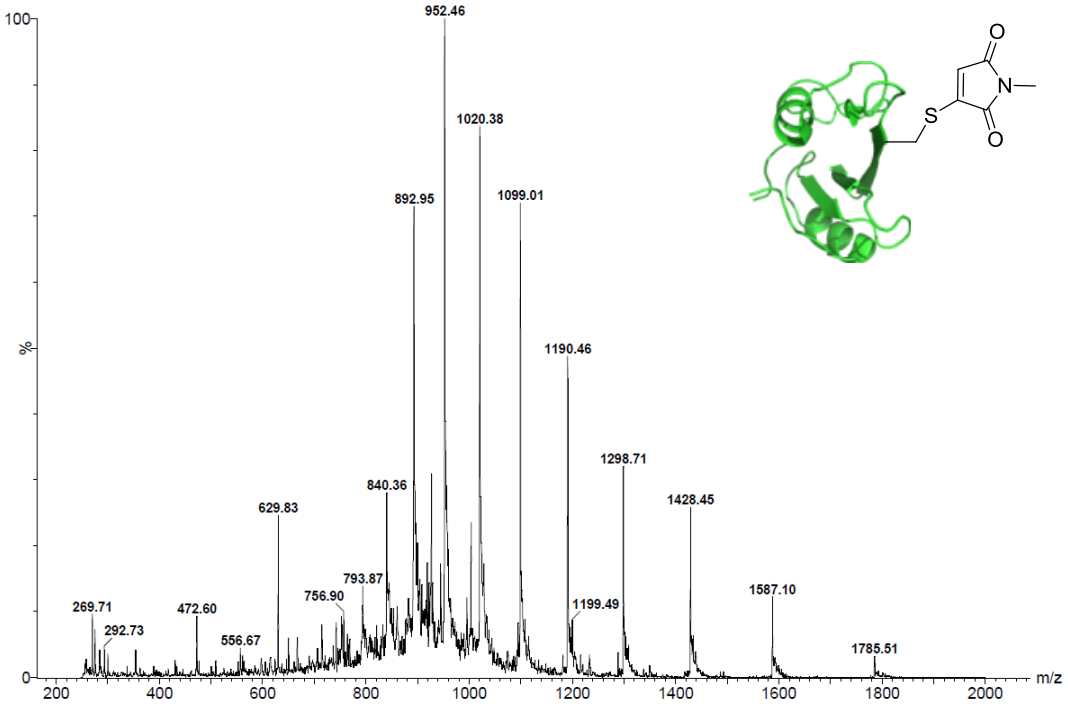
V.11 LC-MS spectra

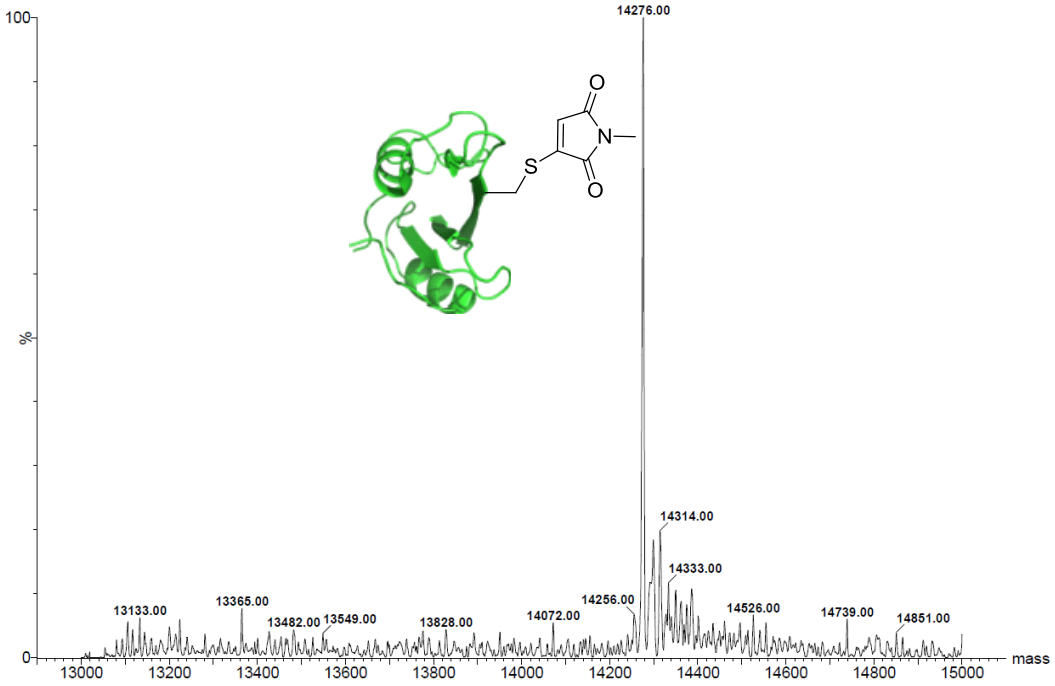
Unreacted Grb2 SH2 (L111C) 26 (eluted at 2.13 min; observed mass: 14169)



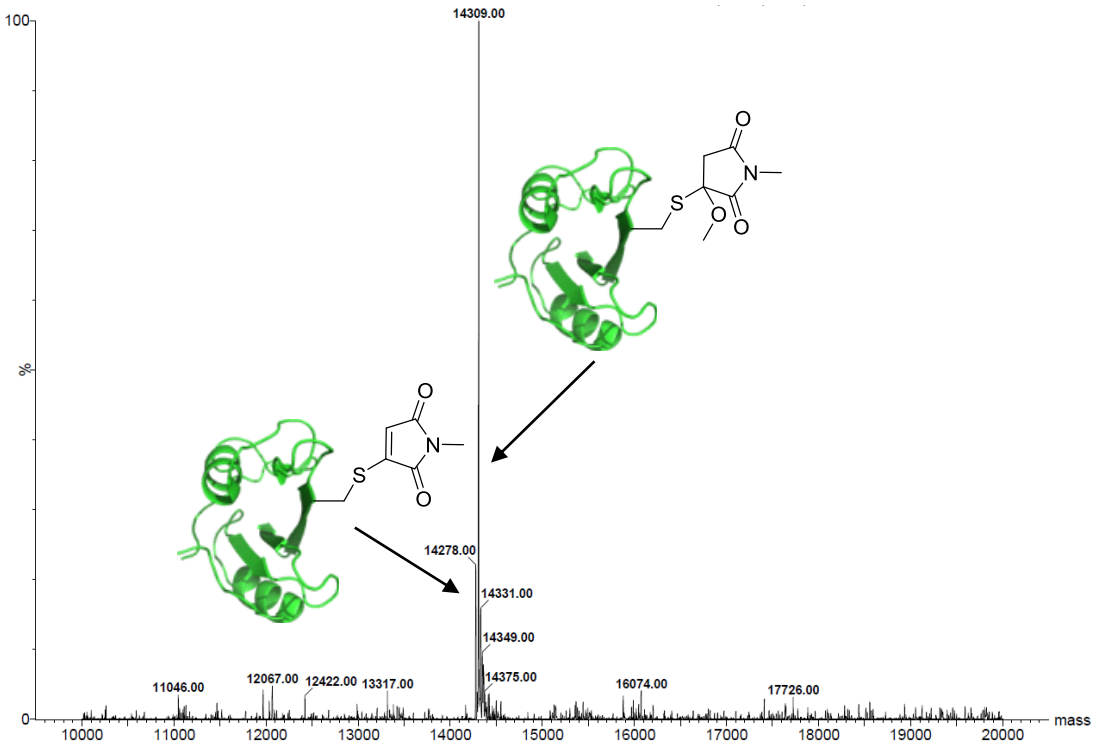


Grb2 SH2 (L111C) thiomaleimide conjugate 113 (eluted at 1.41 min; expected mass: 14282; observed mass: 14276)

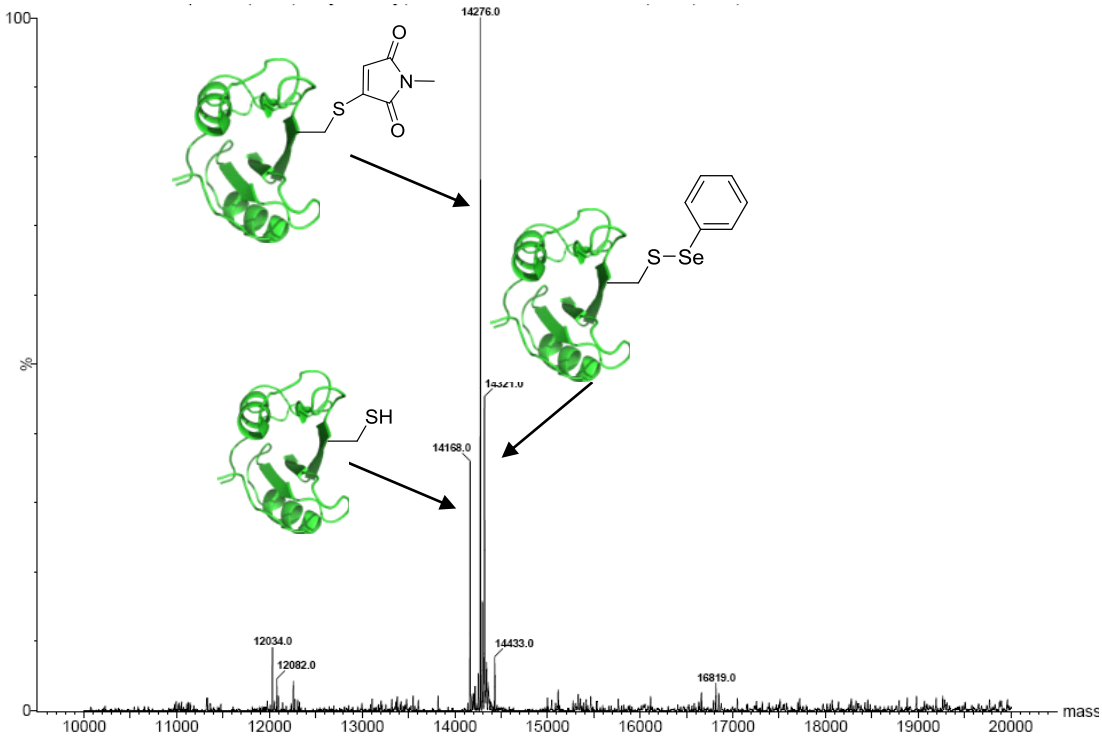




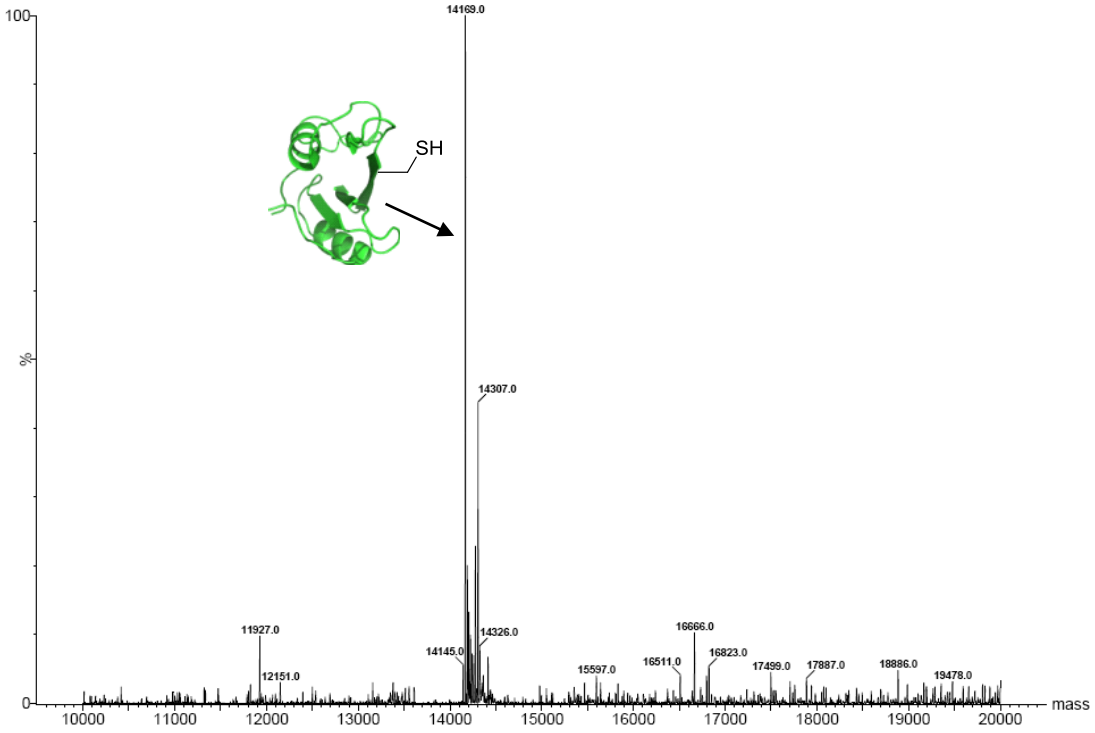
Grb2 + 100 eq. *N*-methyl methoxymaleimide 95 (eluted at 1.41 min)



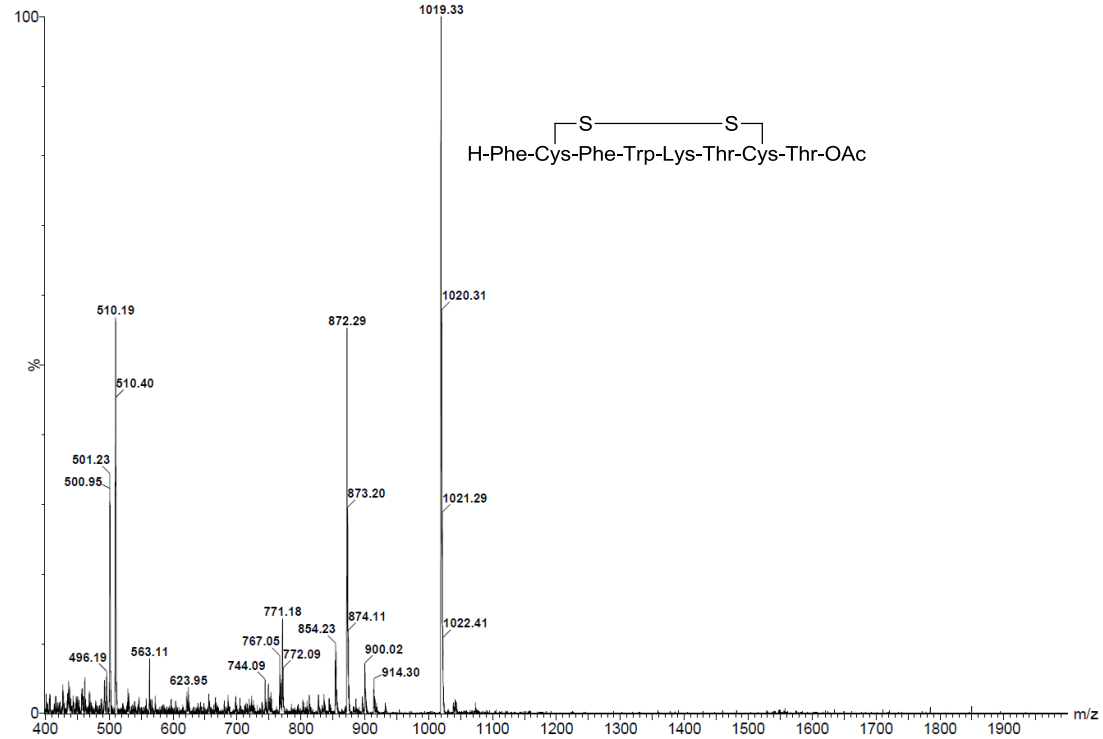
Grb2 + 1 eq. *N*-methyl selenomaleimide 79 (eluted at 1.28 min)



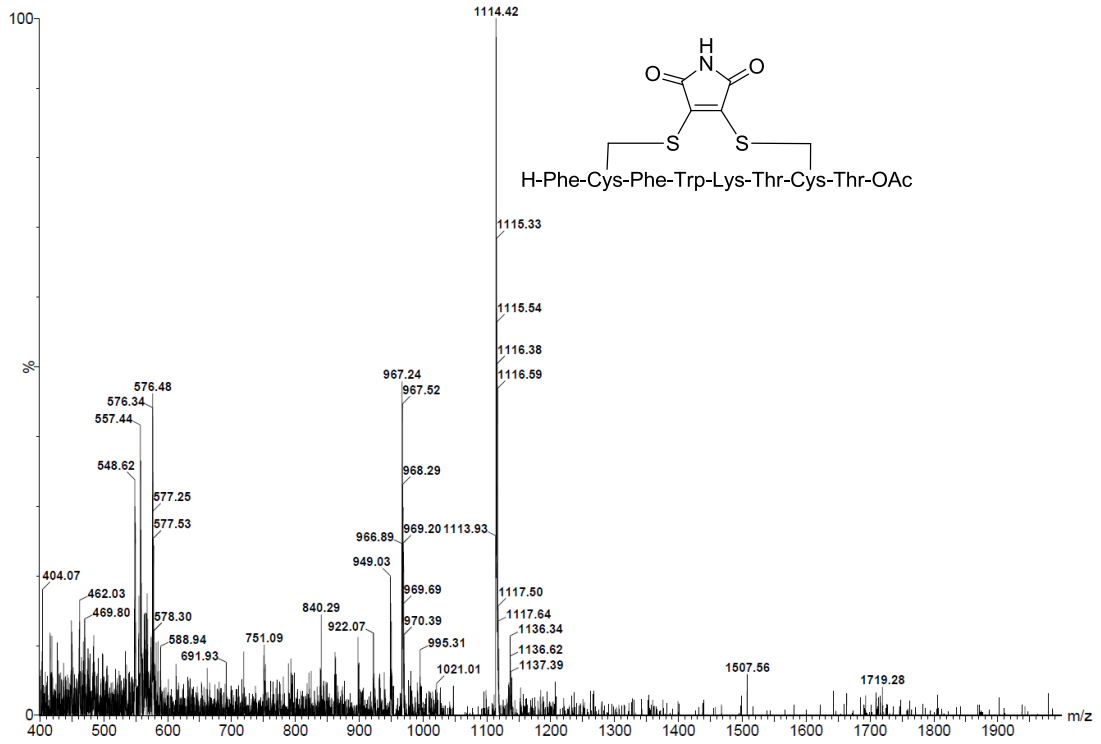
Grb2 + 1 eq. *N*-methyl benzoatemaleimide 82 (eluted at 1.28 min)



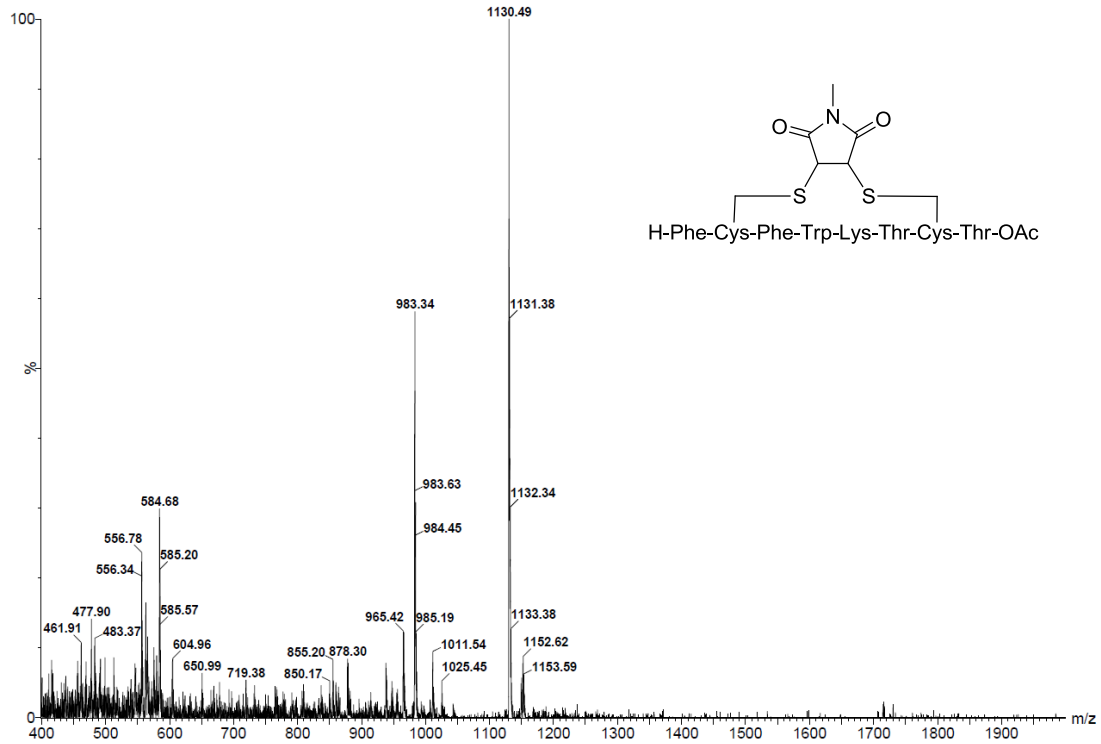
Unreacted octreotide 163 (eluted at 1.73 min; expected mass: 1019; observed mass: 1019)



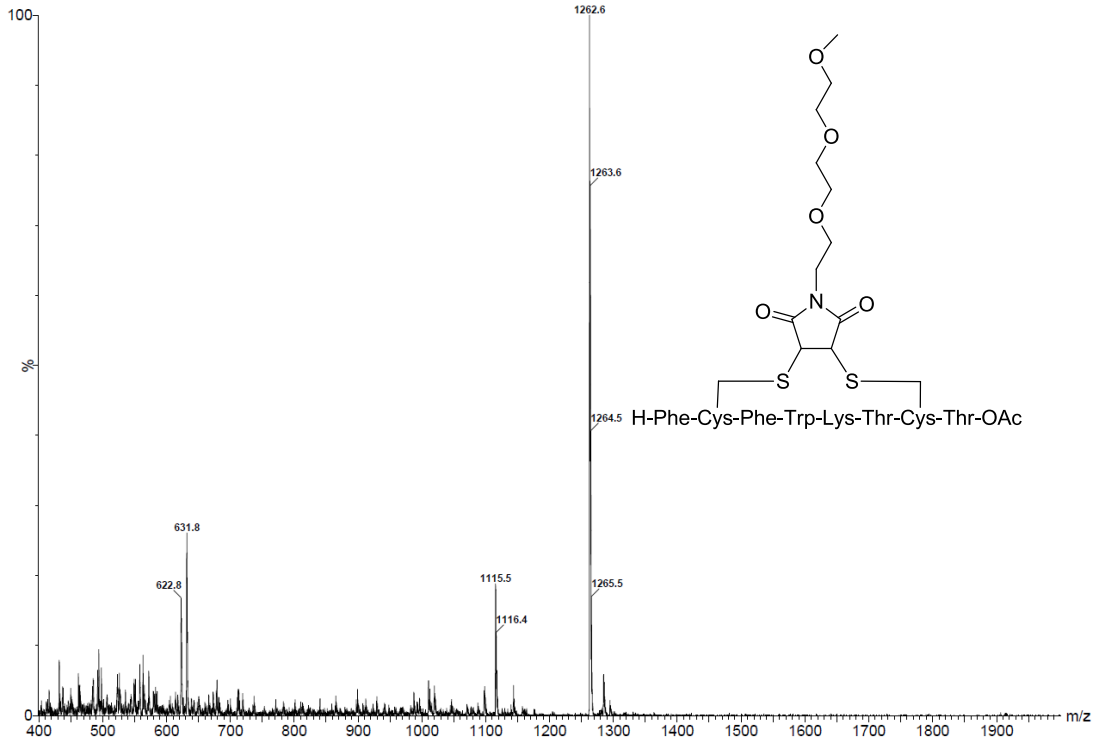
Maleimide bridged octreotide (eluted at 1.15 min; expected mass: 1114; observed mass: 1114)



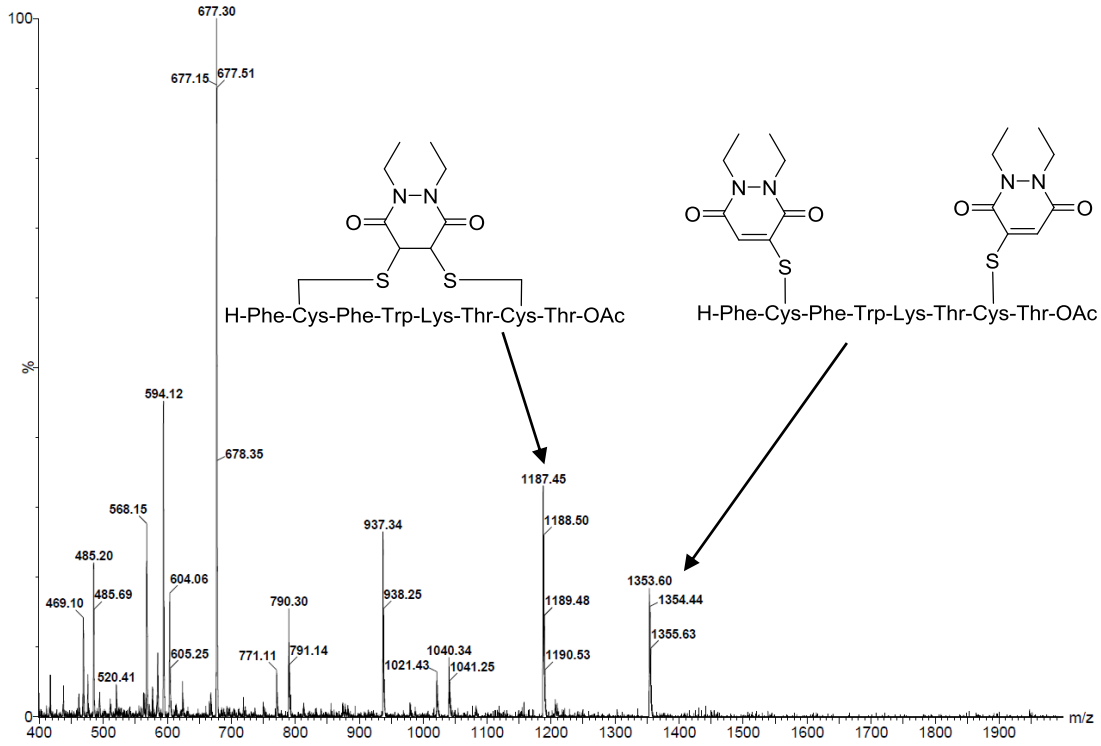
***N*-Methyl succinimide bridged octreotide 164** (eluted at 1.21 min; expected mass: 1130; observed mass: 1130)



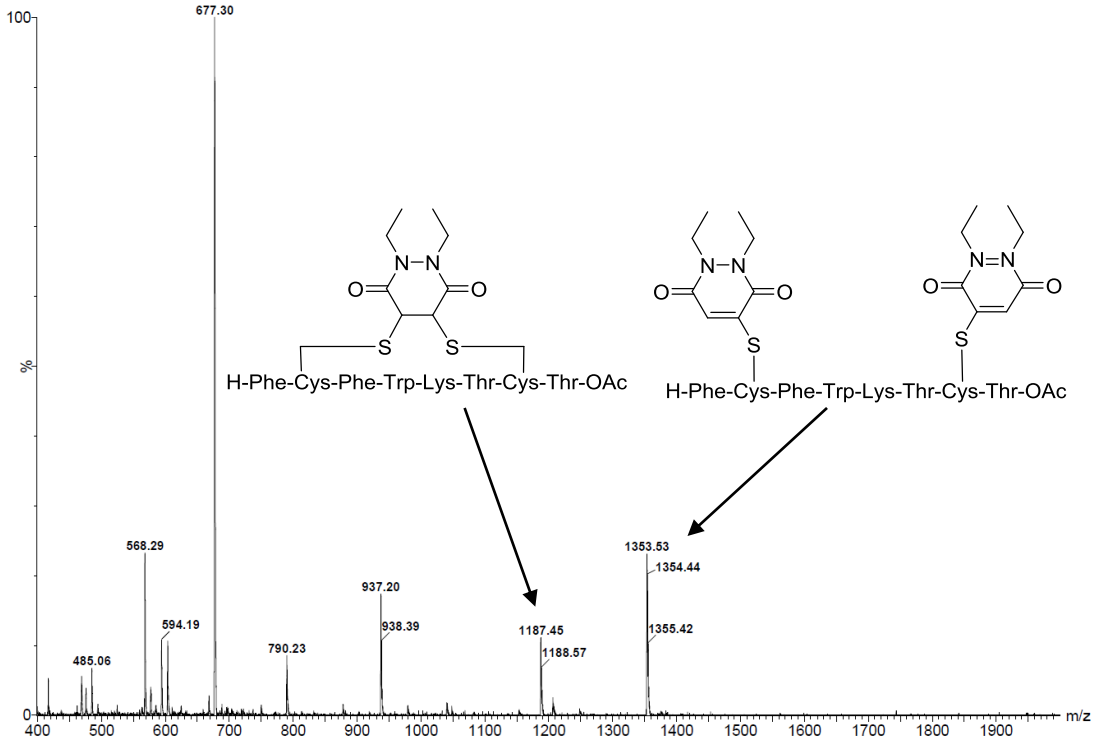
***N*-PEG succinimide bridged octreotide 191** (eluted at 2.7 min; expected mass: 1262; observed mass: 1262)



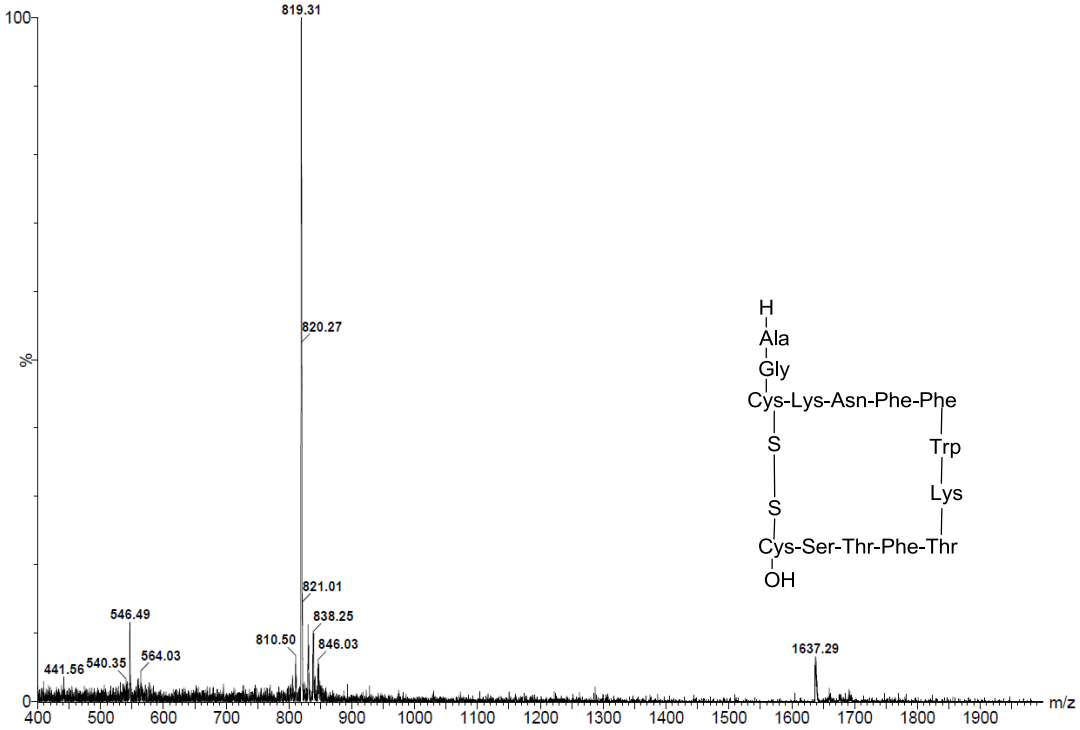
Octreotide + 5 eq. *N,N'*-diethyl bromopyridazinedione 187 (**195,** eluted at 1.28 min: expected mass: 1187; observed mass: 1187; **196,** eluted at 1.36 min: expected mass: 1353; observed mass: 1354)



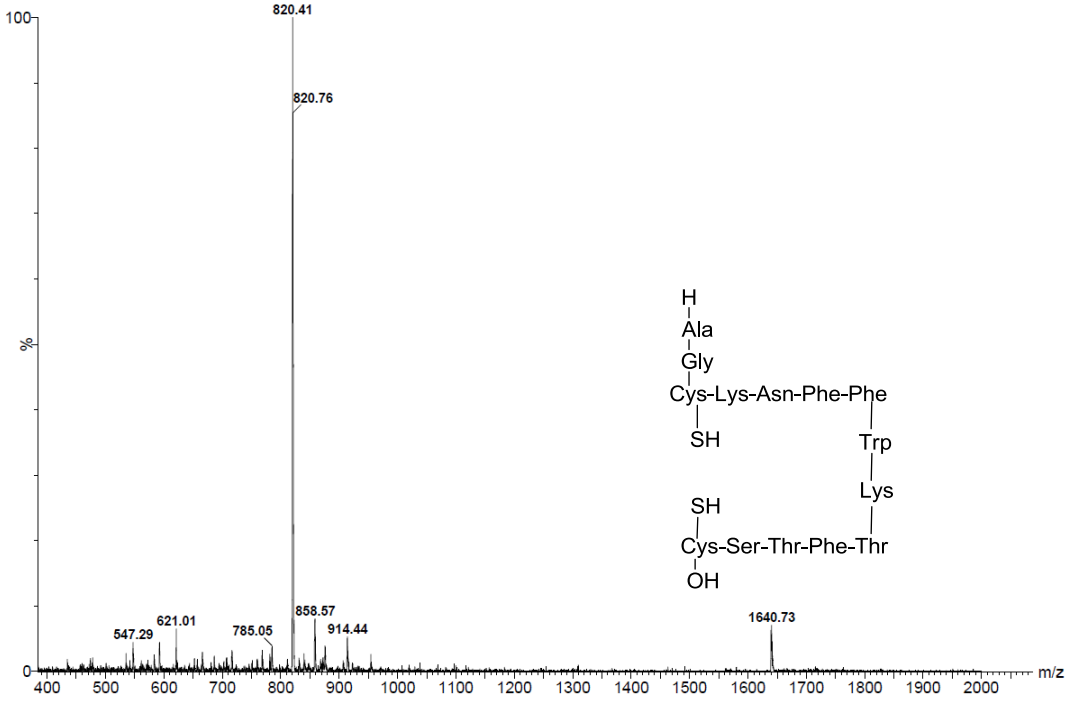
Octreotide + 10 eq. *N,N'*-diethyl bromopyridazinedione 187 (**195,** eluted at 1.28; expected mass: 1187; observed mass: 1187; **196,** eluted at 1.36 min; expected mass: 1353; observed mass: 1354)



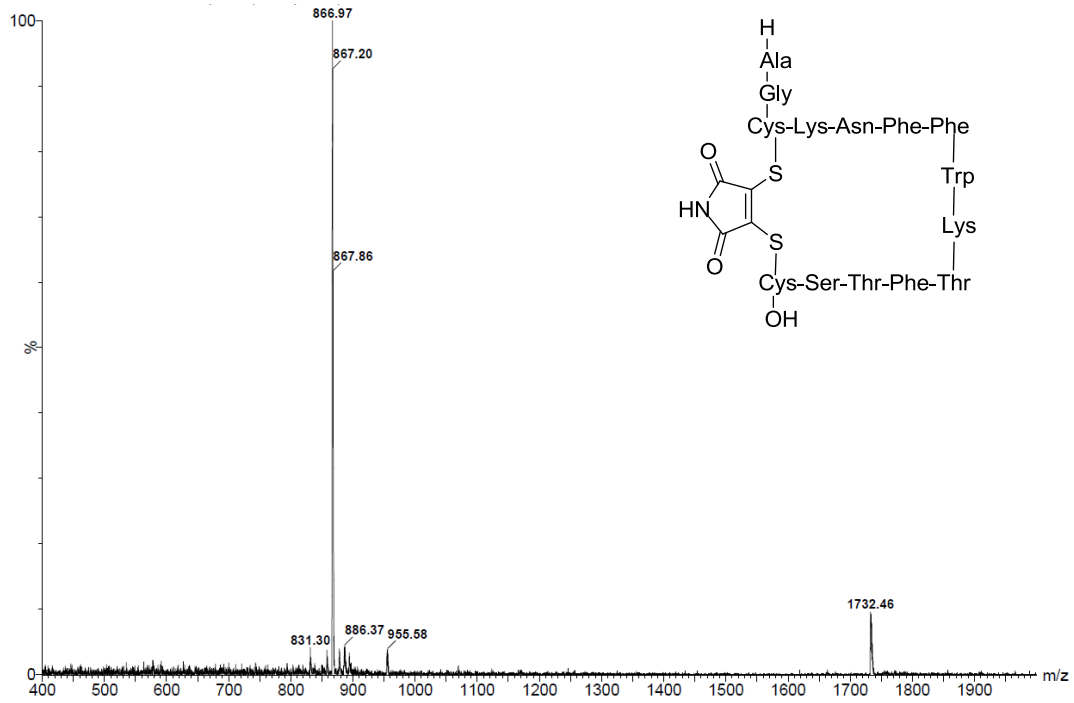
Unreacted somatostatin 29 (eluted at 1.24 min; expected mass: 1637; observed mass: 1637)



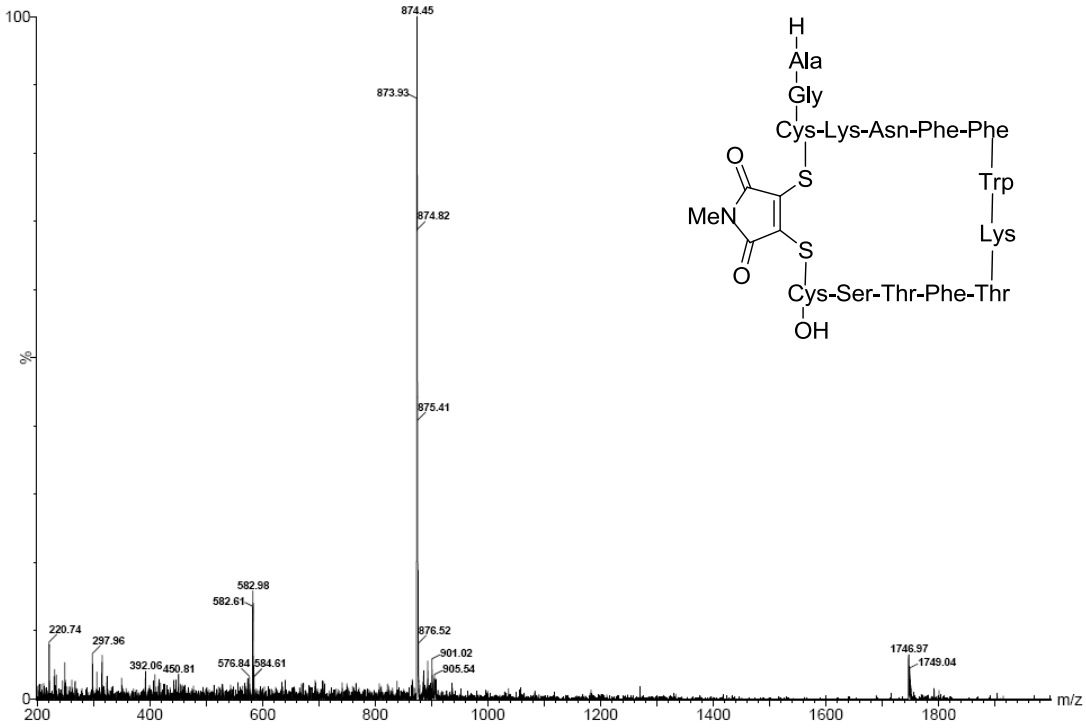
Reduced somatostatin 118 (eluted at 1.26 min; expected mass: 1639; observed mass: 1641)



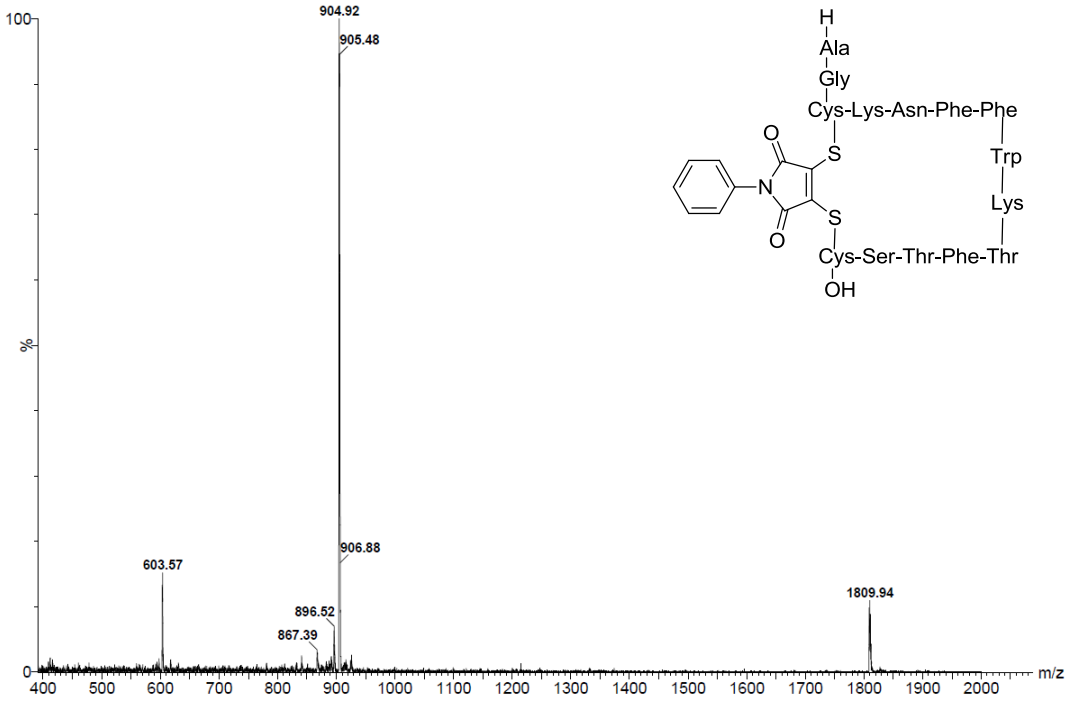
Maleimide bridged somatostatin 119 (eluted ar 1.27 min; expected mass: 1732; observed mass: 1732)



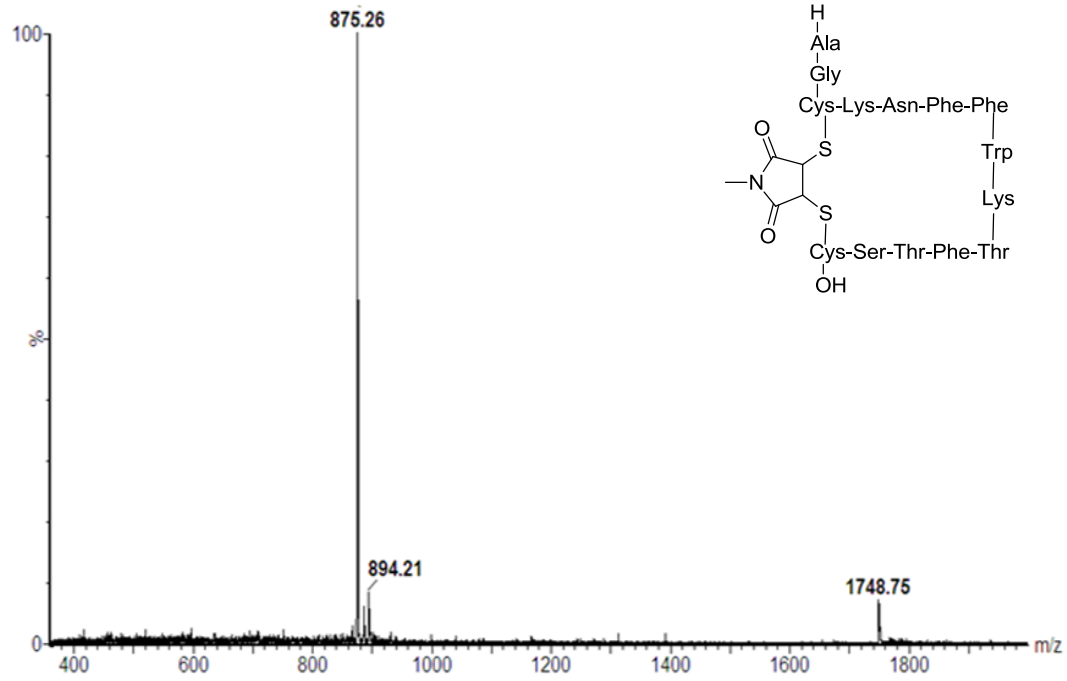
***N*-methyl maleimide bridged somatostatin 120** (eluted at 1.30 min; expected mass: 1746; observed mass: 1747)



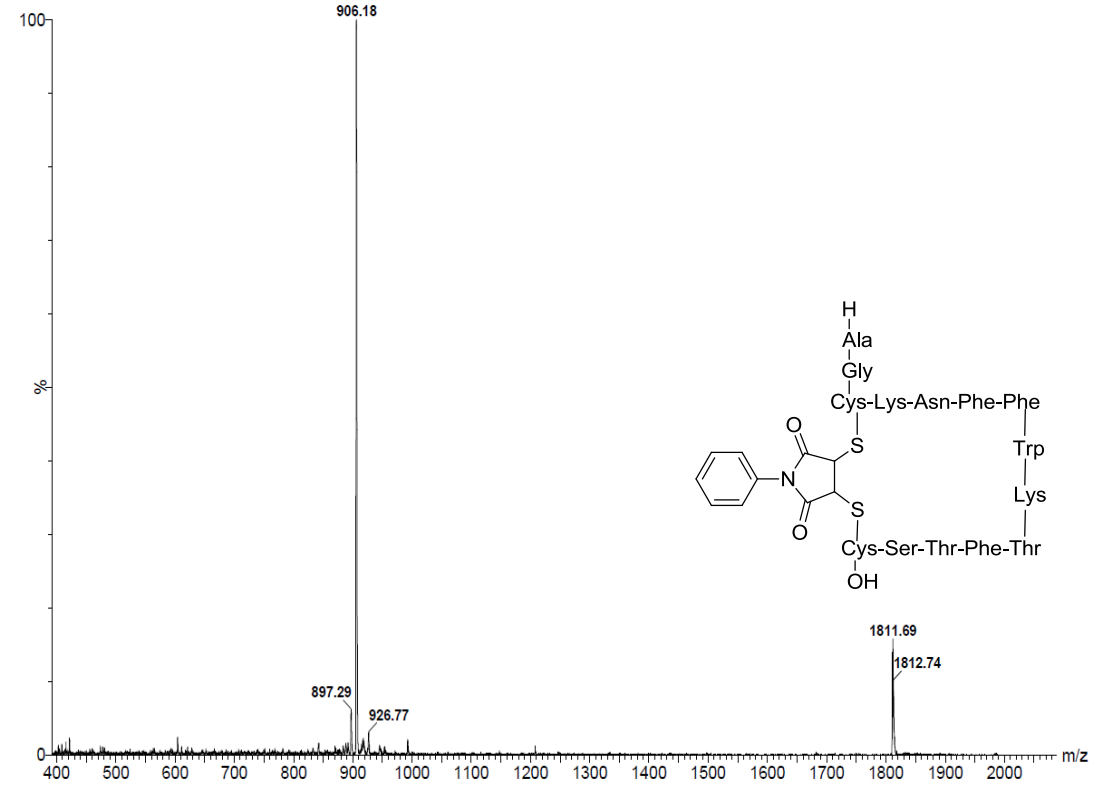
***N*-Phenyl maleimide bridged somatostatin 175** (eluted at 1.81 min; expected mass: 1808; observed mass: 1810)



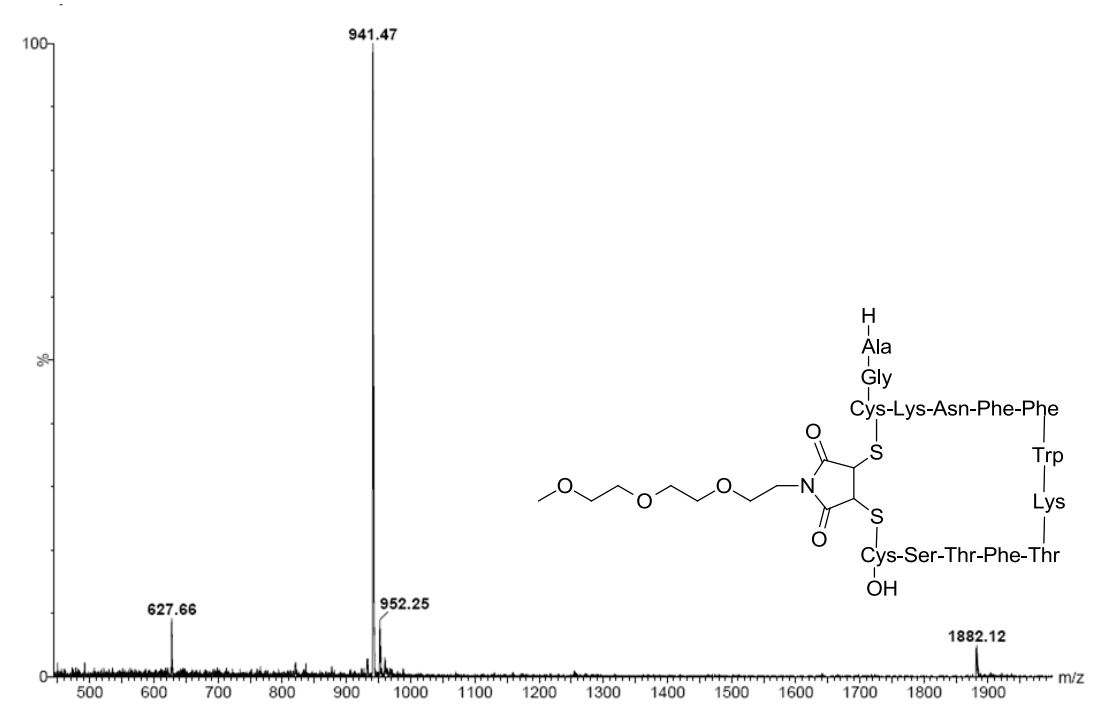
***N*-Methyl succinimide bridged somatostatin 128** (eluted at 1.50 min; expected mass: 1748; observed mass: 1749)



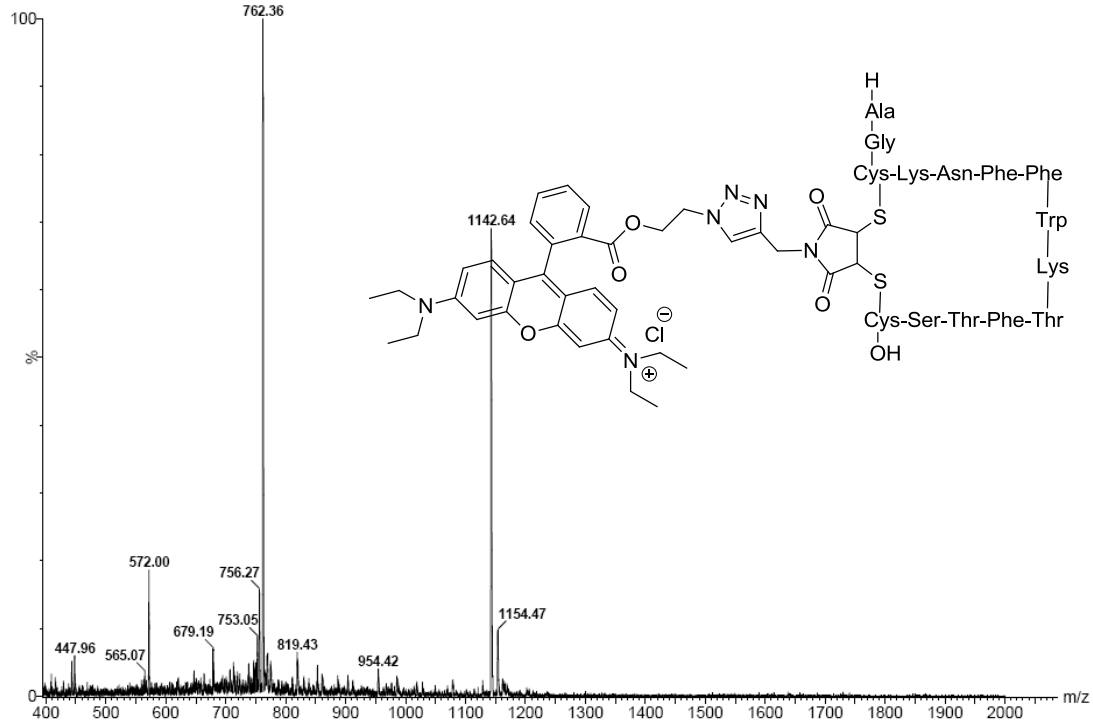
***N*-Phenyl succinimide bridged somatostatin 174** (eluted at 1.50 min; expected mass: 1810; observed mass: 1812)

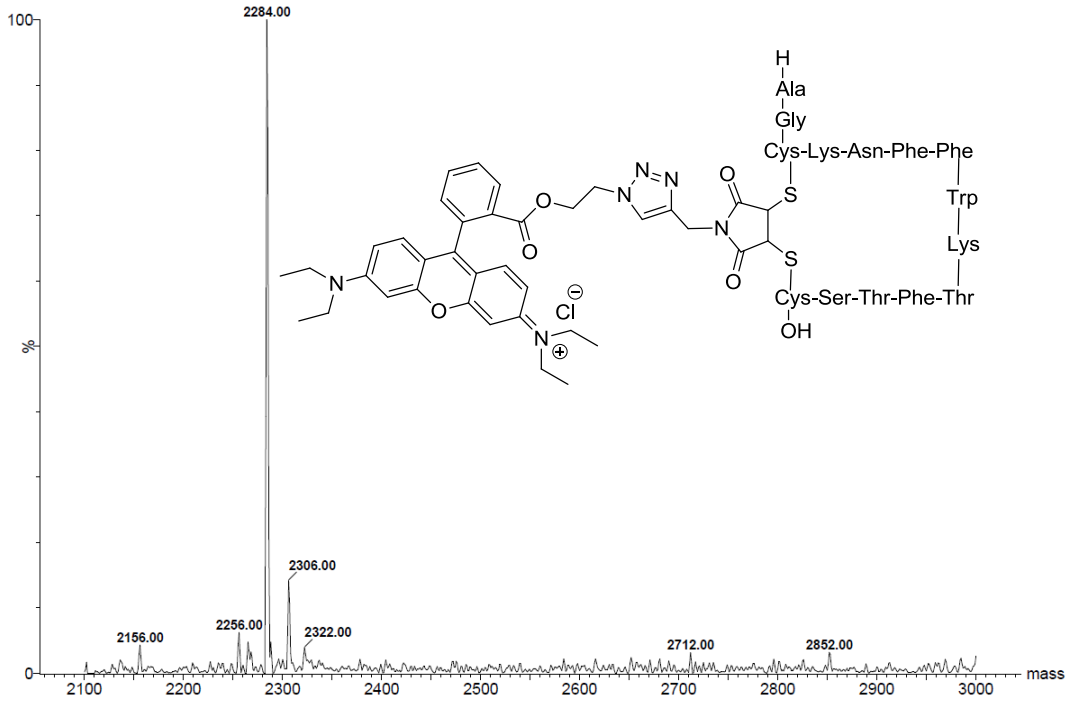


***N*-PEG succinimide bridged somatostatin 161** (eluted at 1.54 min; expected mass: 1880; observed mass: 1882)

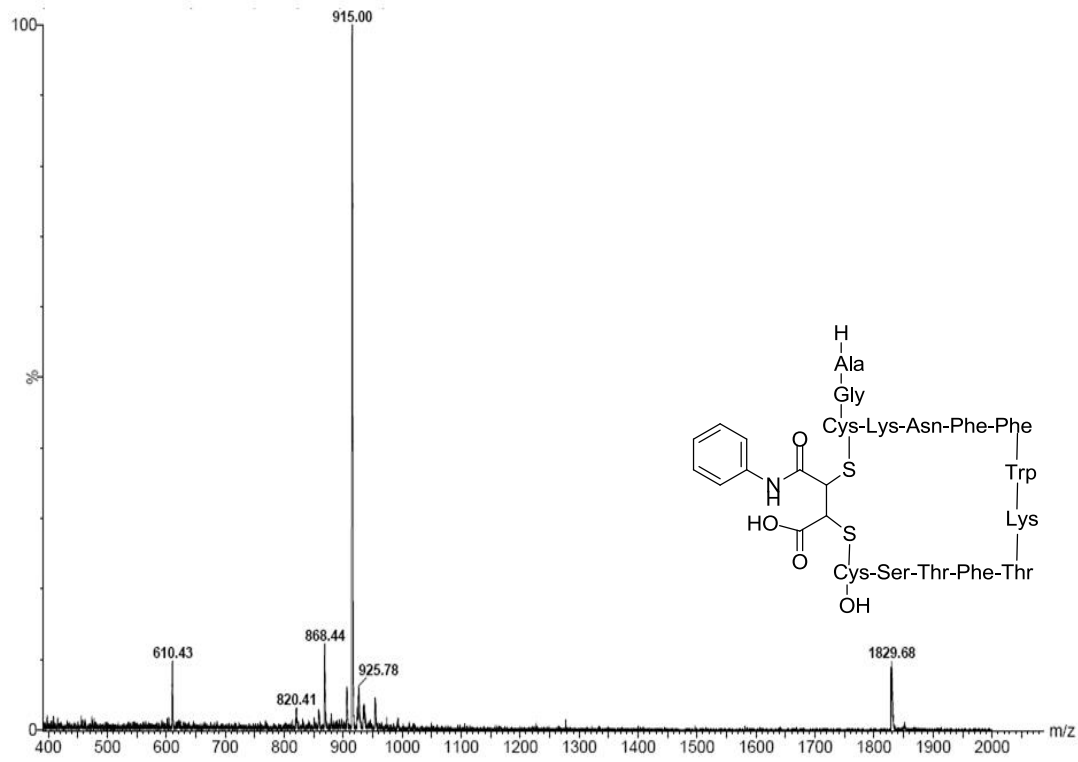


Rhodamine succinimide bridged somatostatin 162 (eluted at 1.88 min; expected mass: 2283; observed mass: 2284)

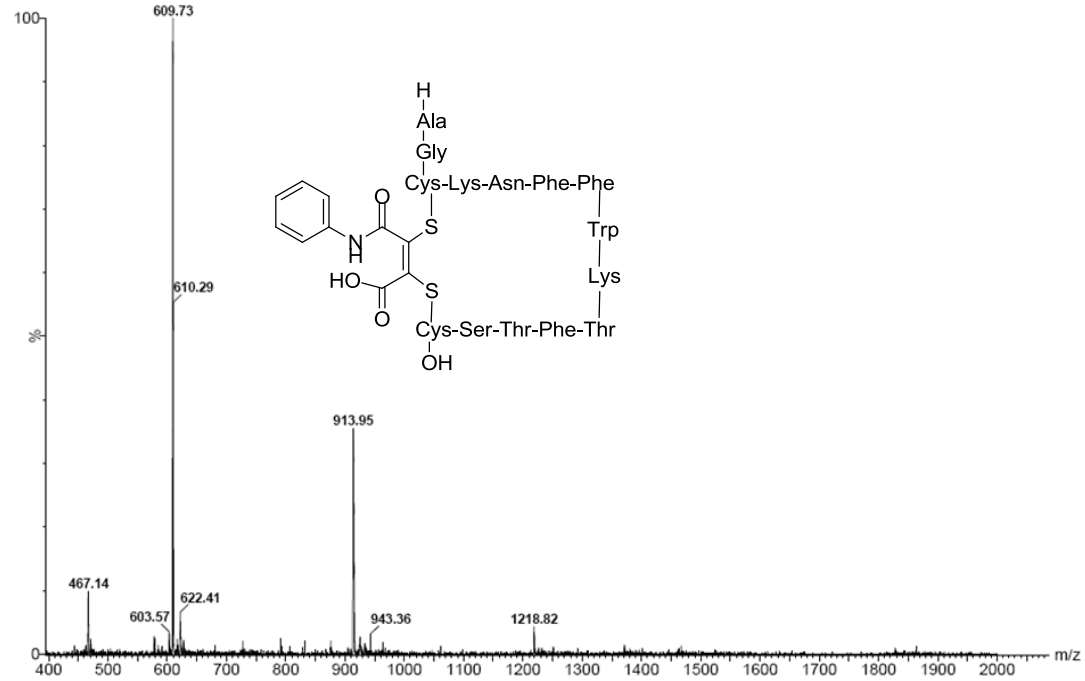




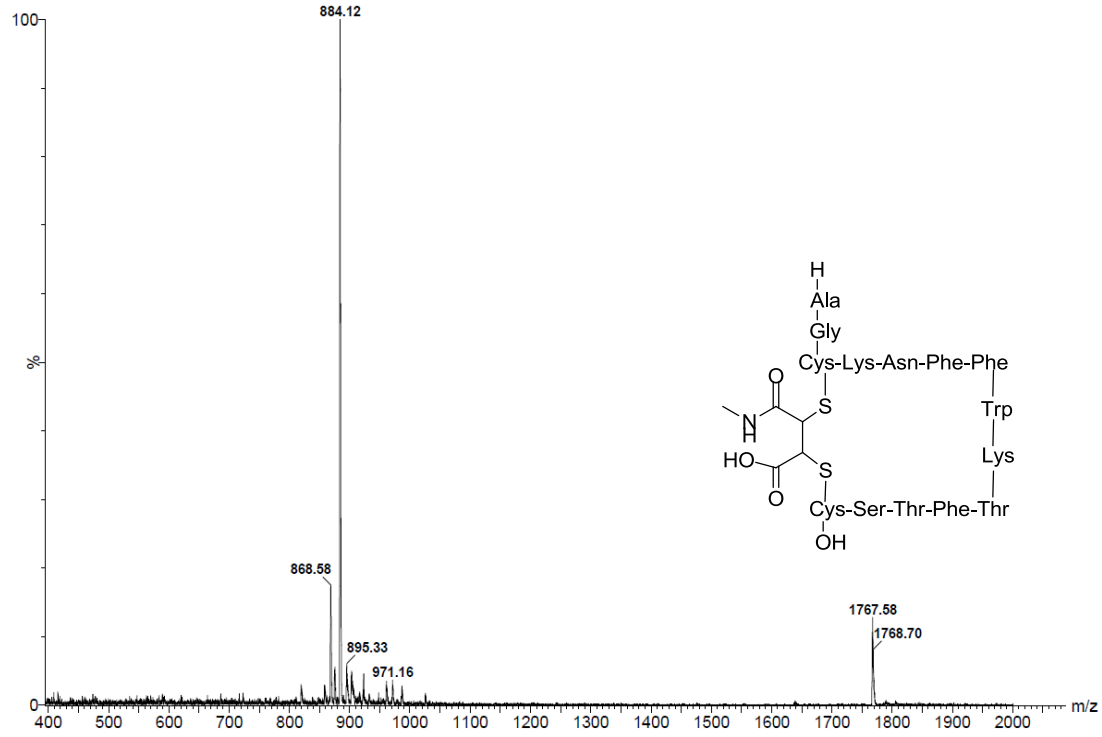
Hydrolysed *N*-phenyl succinimide bridged somatostatin (eluted at 1.68 min; expected mass: 1830; observed mass: 1830)



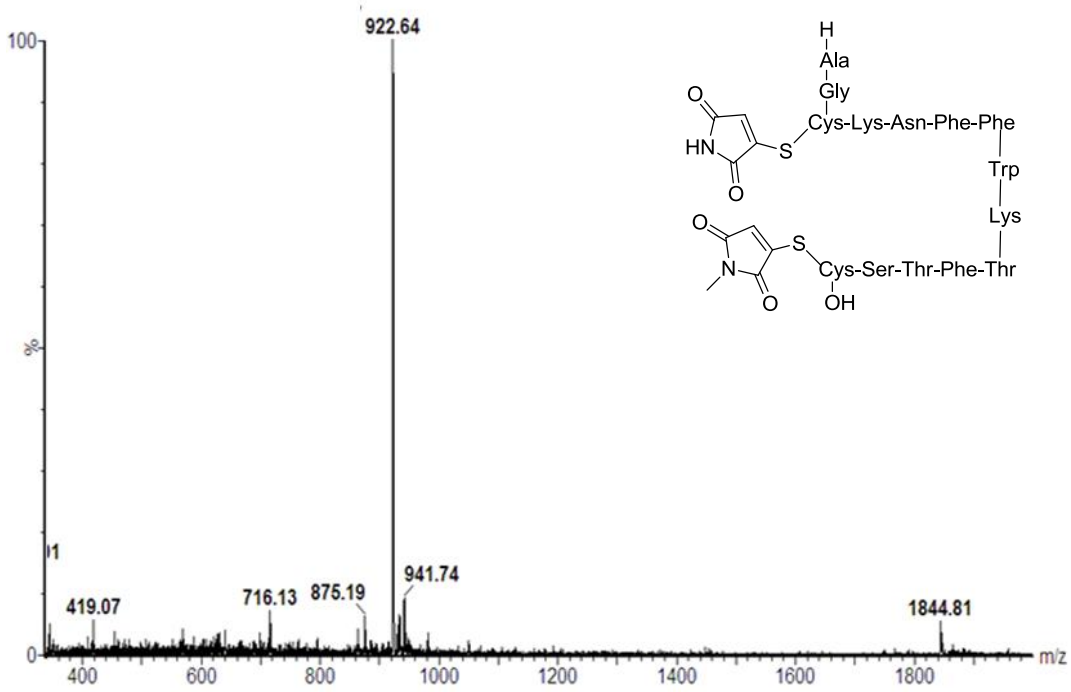
Hydrolysed *N*-phenyl maleimide bridged somatostatin (eluted at 1.73 min; expected mass: 1827; observed mass: 914 (M+2/2))



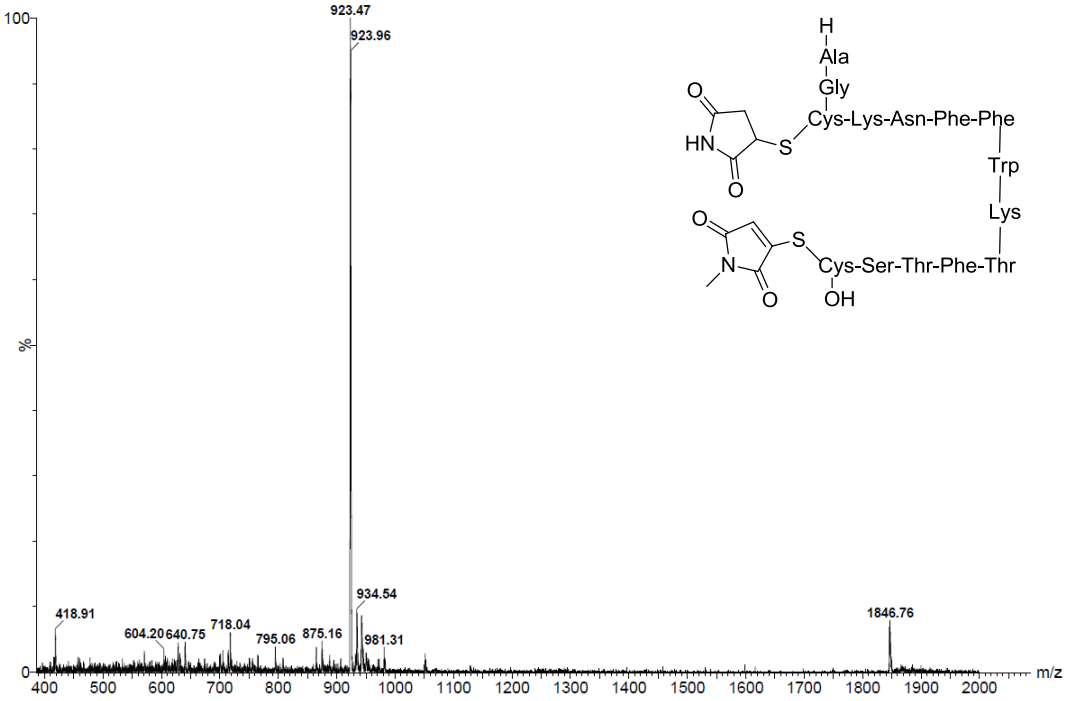
Hydrolysed *N*-methyl succinimide bridged somatostatin (eluted at 1.55 min; expected mass: 1766; observed mass: 1768)



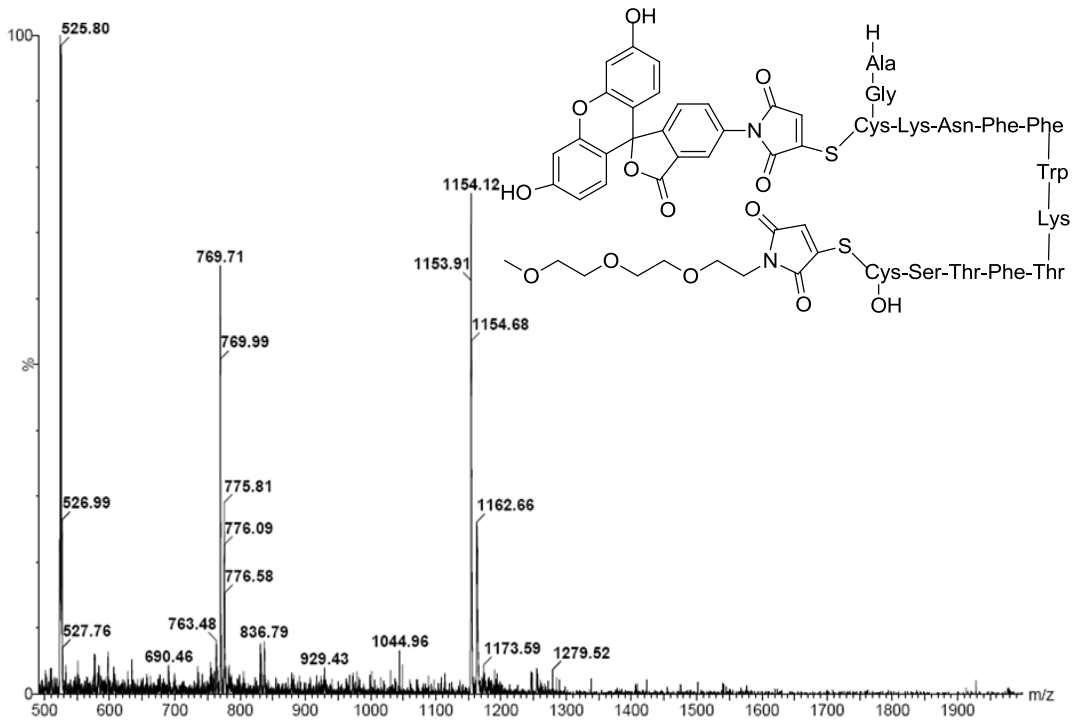
Dual labelled somatostatin conjugate 177 (eluted at 1.49 min; expected mass: 1845; observed mass: 1845)

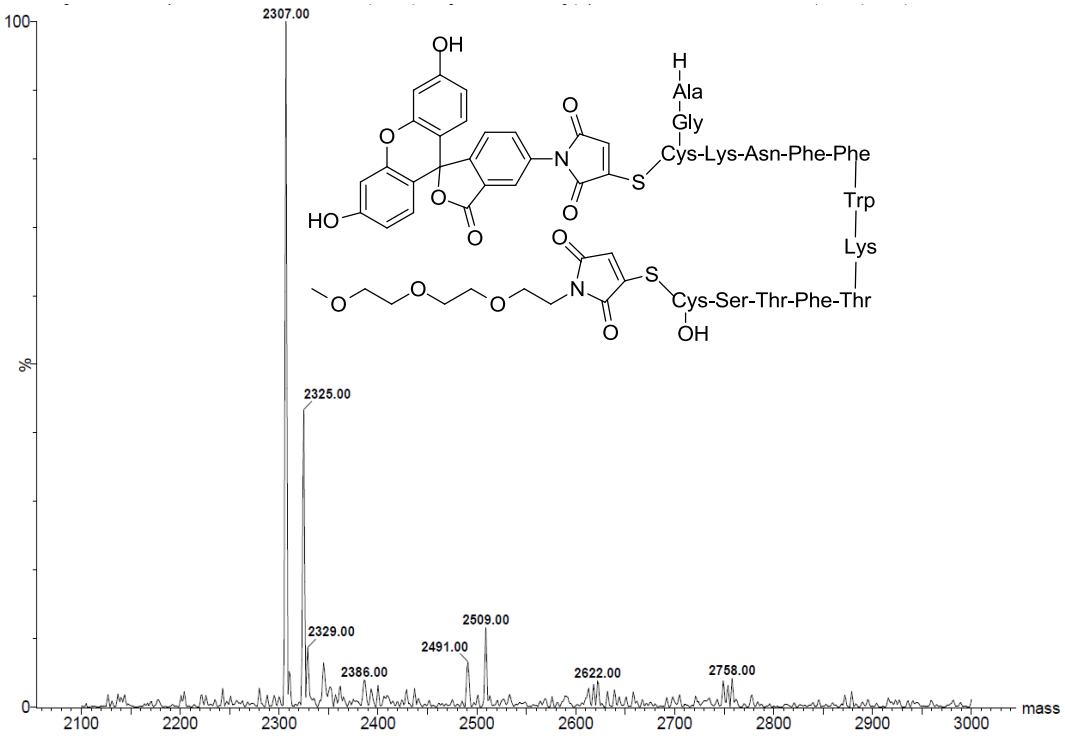


Dual labelled somatostatin conjugate with a maleimide and succinimide tag 178 (eluted at 1.45 min; expected mass: 1845; observed mass: 1847)

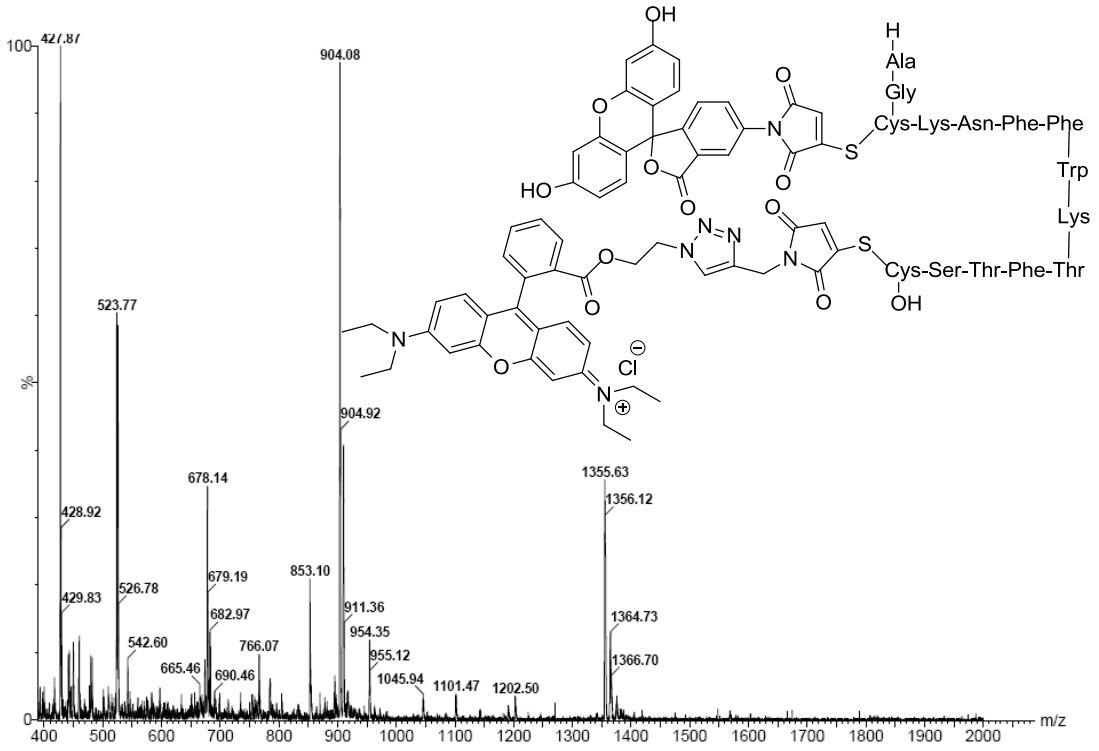


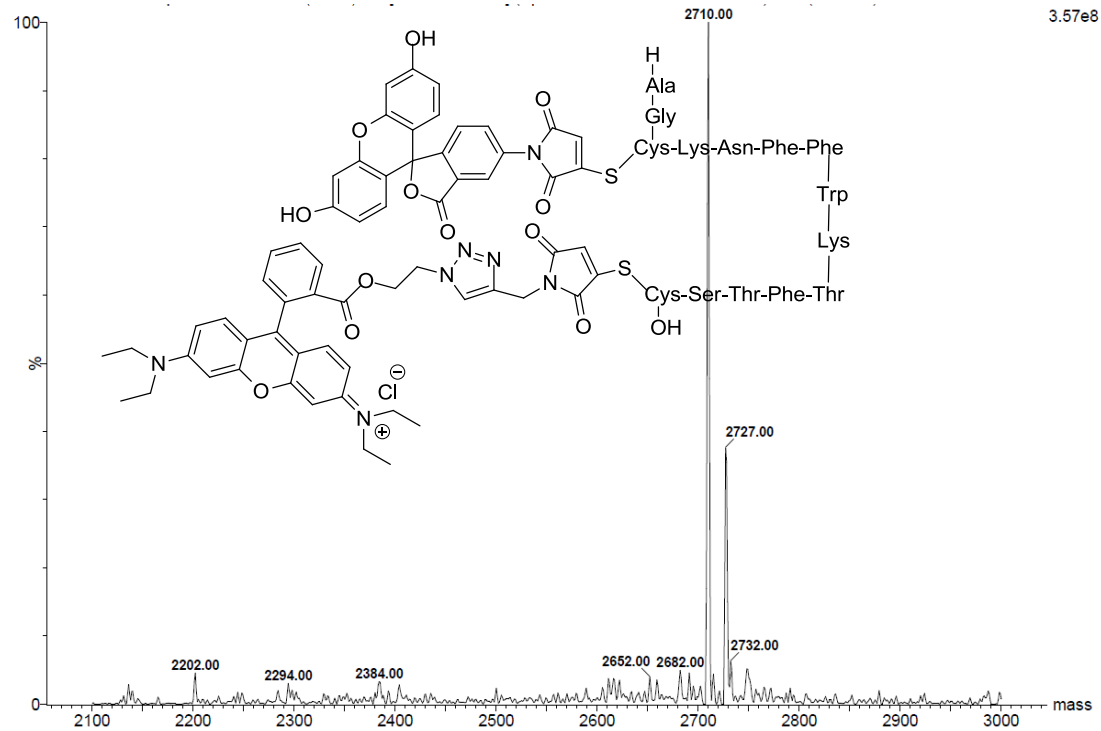
Fluorescein-PEG dual labelled somatostatin conjugate 180 (eluted at 1.73 min; expected mass: 2305; observed mass: 2307)



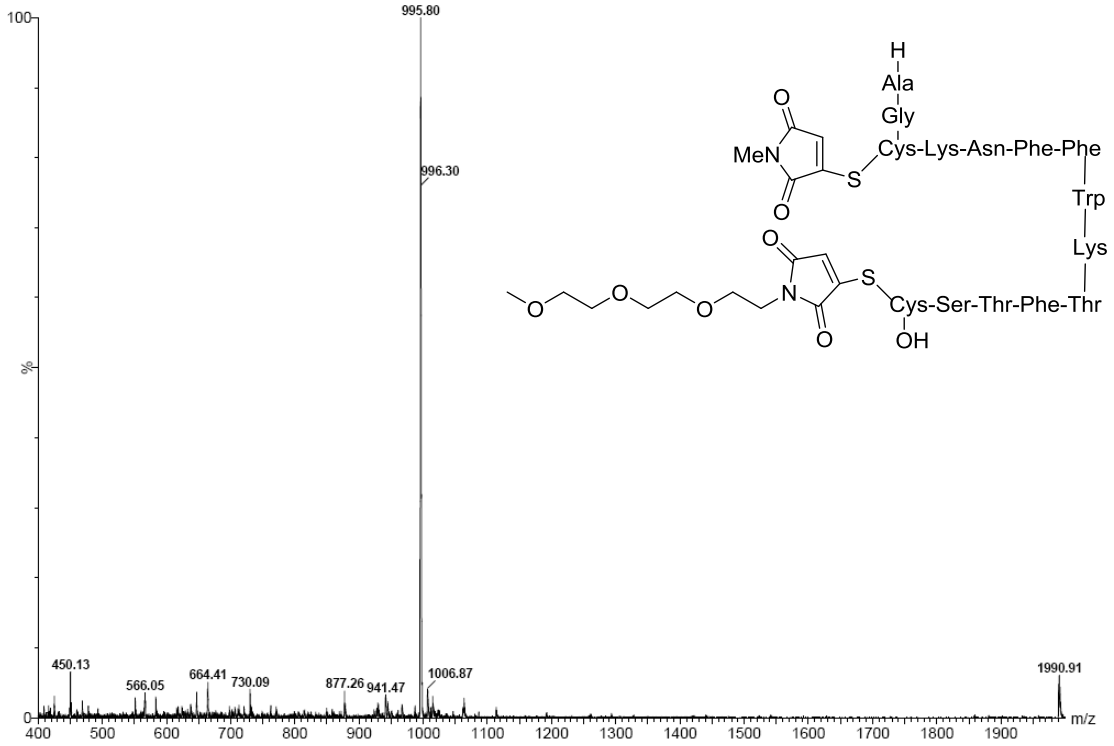


Rhodamine-fluorescein dual labelled somatostatin conjugate 182 (eluted at 2.33; expected mass: 2709; observed mass: 2710)

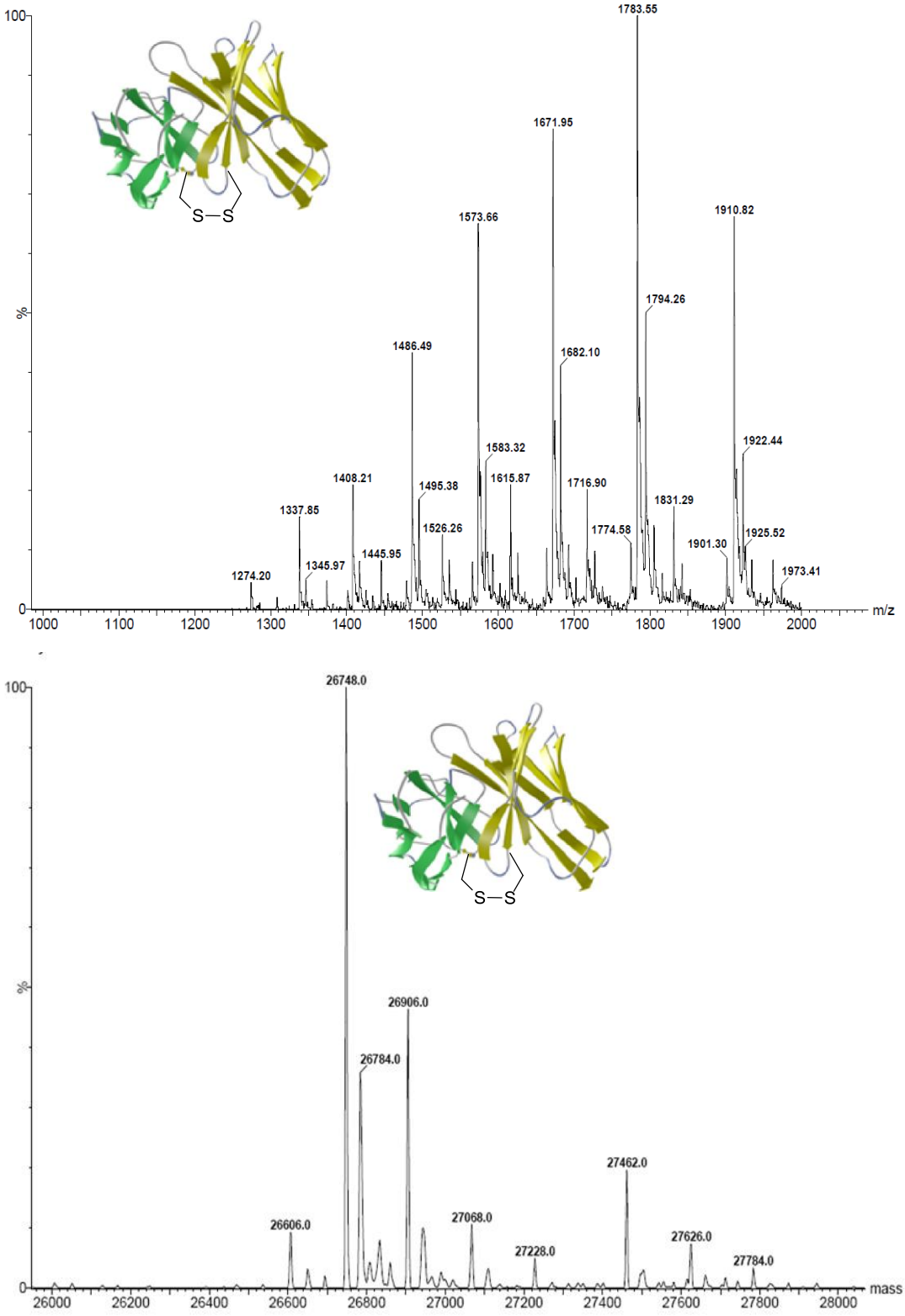




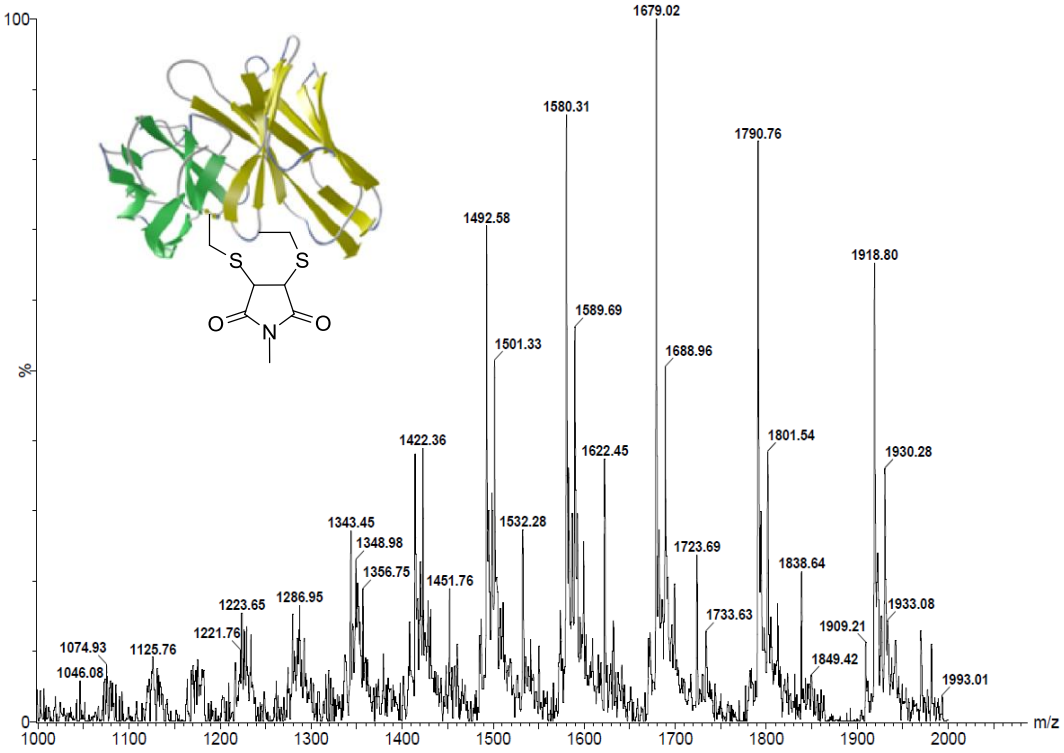
PEG-methyl dual labelled somatostatin conjugate 25 (expected mass: 1989; observed mass: 1991)

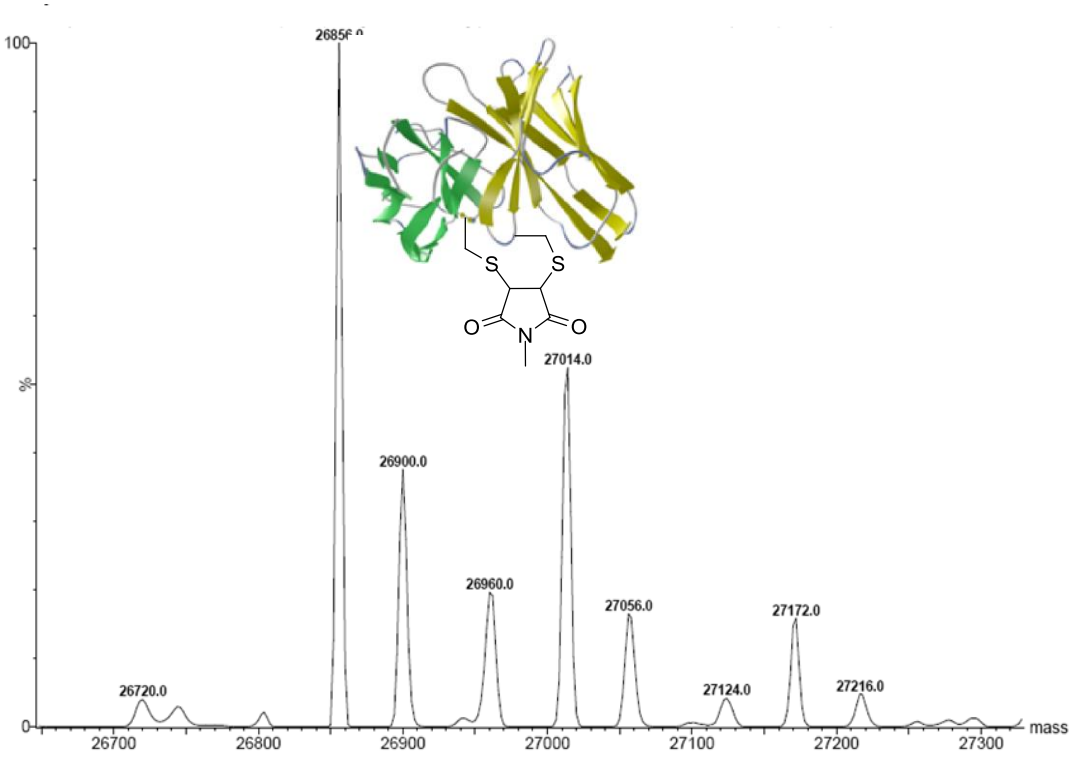


Unreacted anti-CEA ds-scFv 33 (eluted at 1.36-1.75 min; observed mass: 26748)

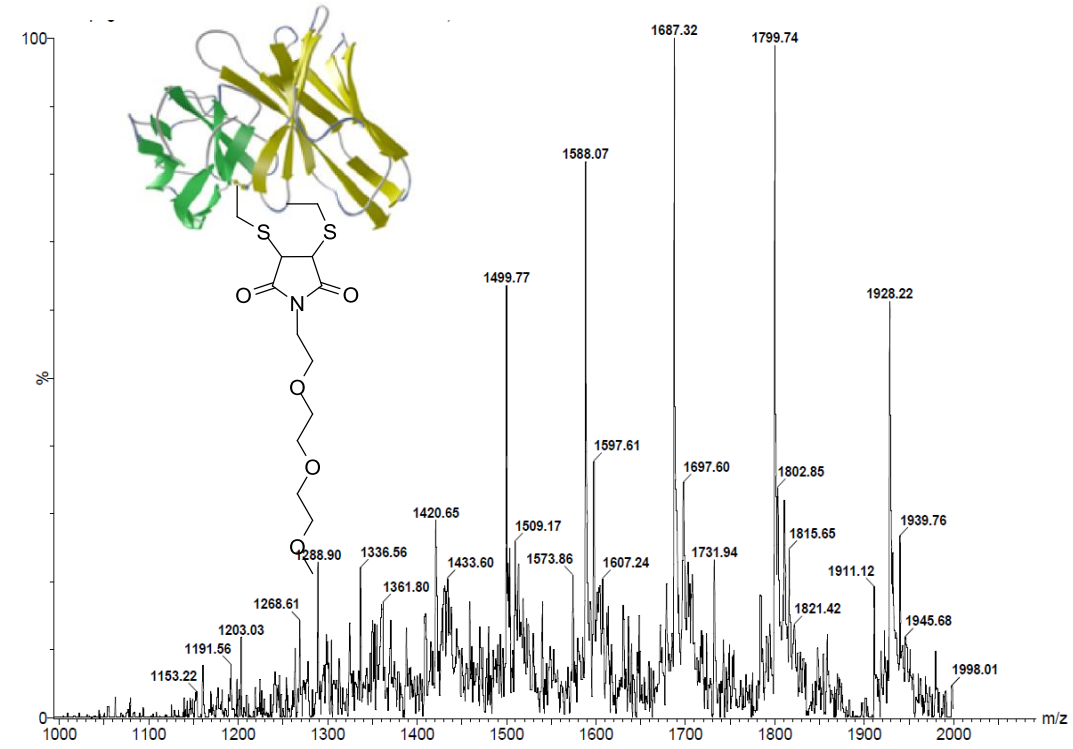


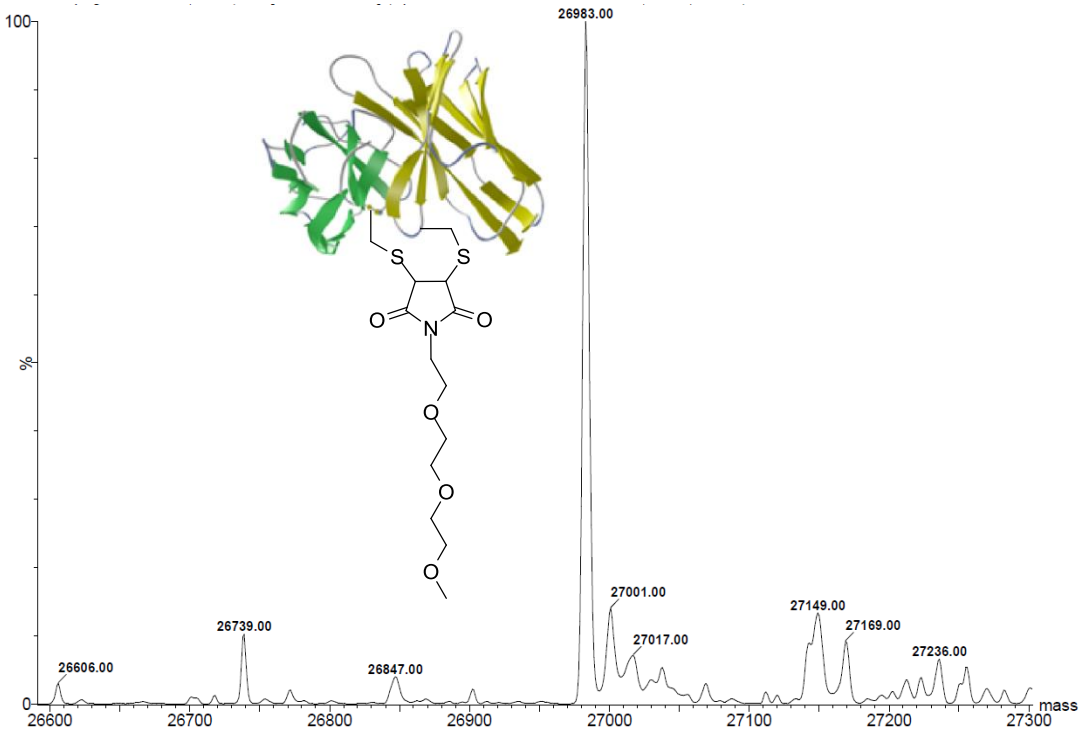
***N*-Methyl succinimide bridged anti-CEA ds-scFv 169** (eluted at 1.38 min; expected mass: 26859; observed mass: 26856)



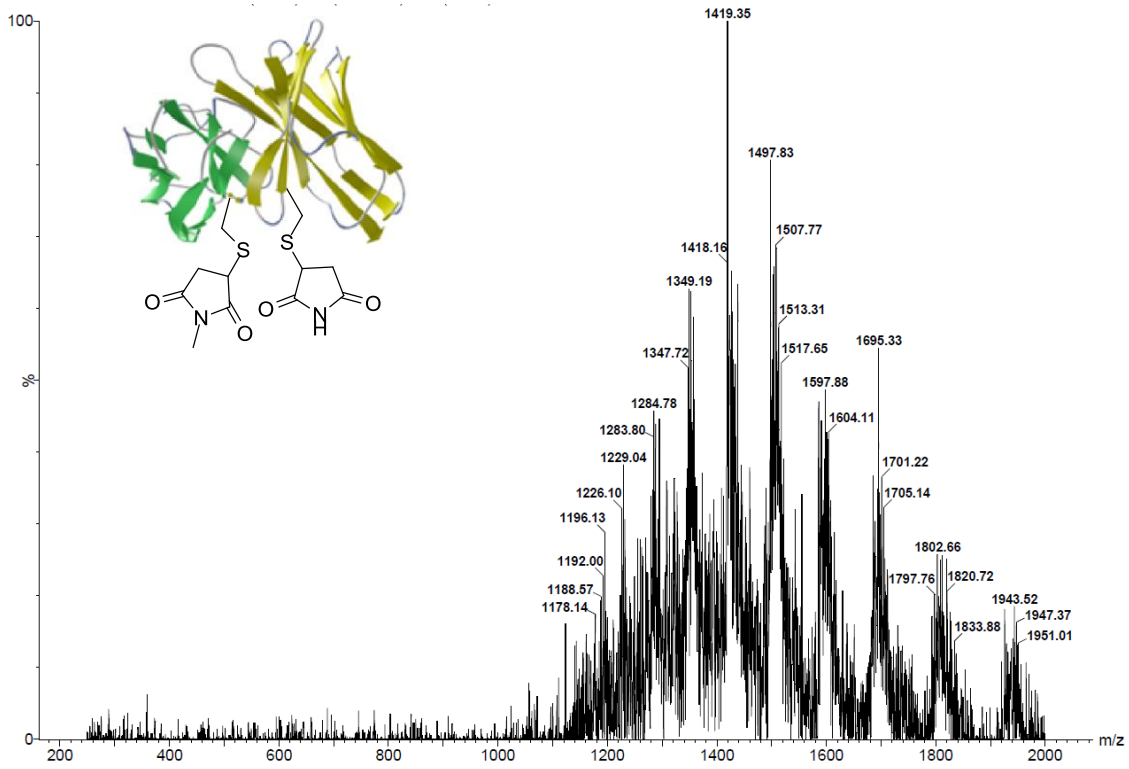


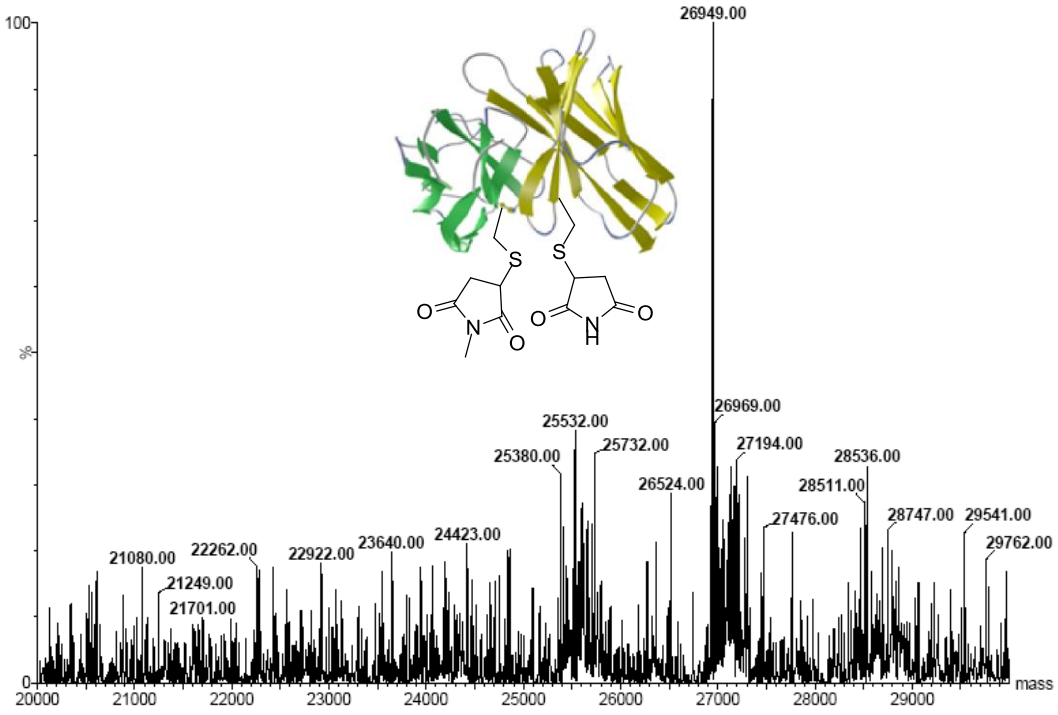
***N*-PEG succinimide bridged anti-CEA ds-scFv 170** (eluted at 1.50 min; expected mass: 26991; observed mass: 26983)



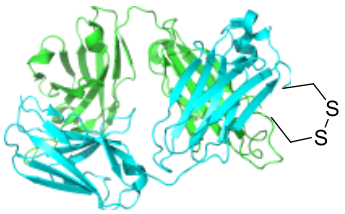


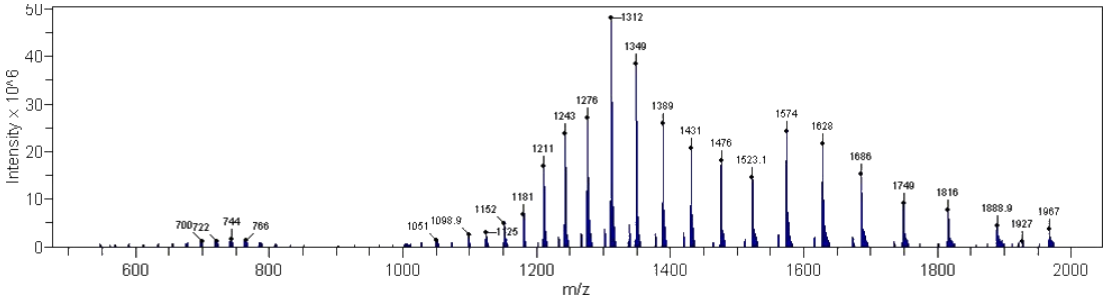
Dual labelled ds-scFv conjugate 203 (eluted at 1.49 min; expected mass: 26953; observed mass: 26949)

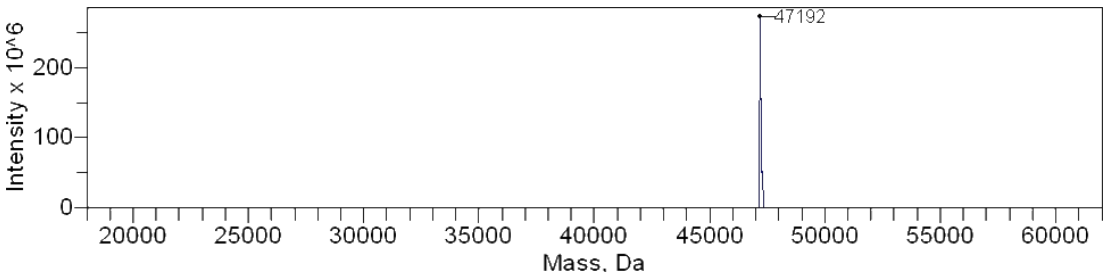




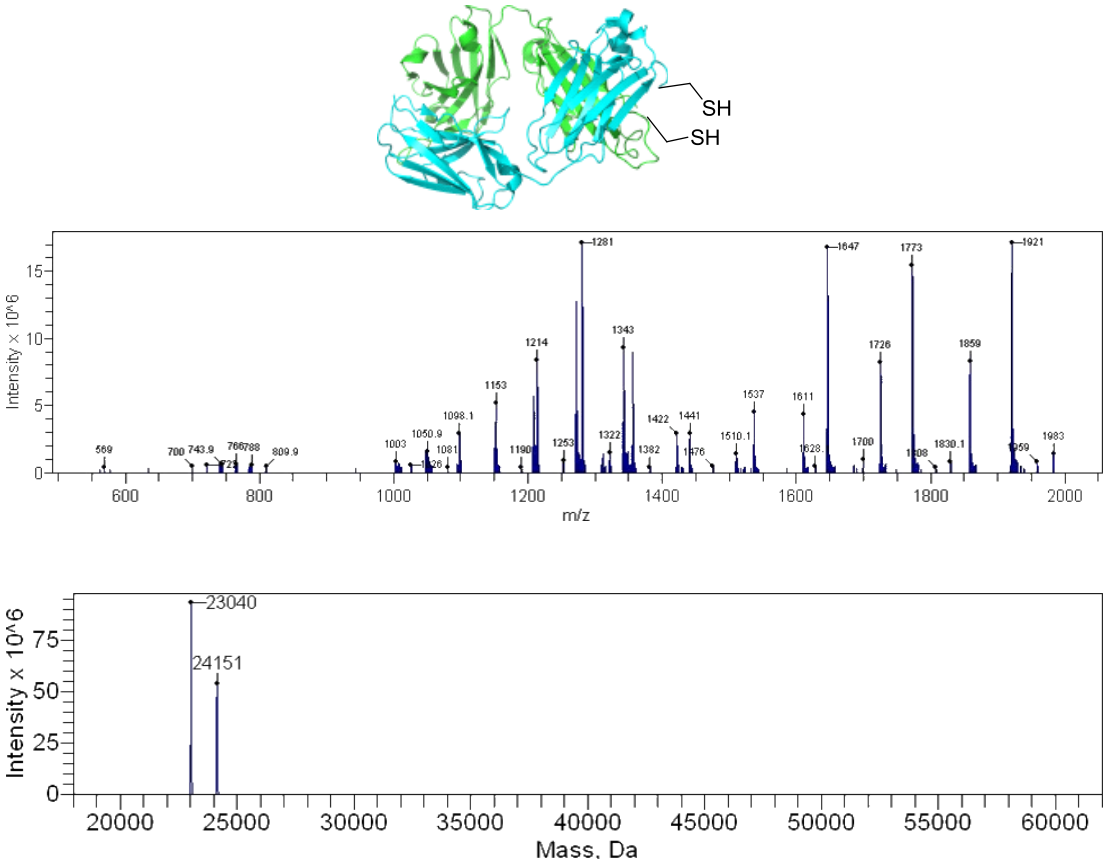
Unreacted Rituximab Fab 165 (eluted at 3.74 min; observed mass: 47192)



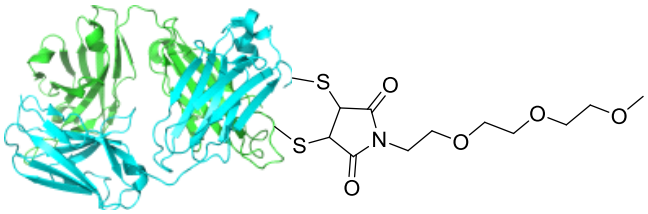


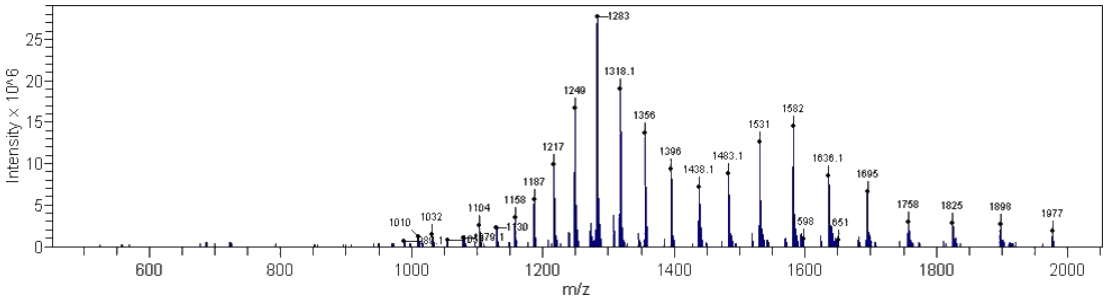


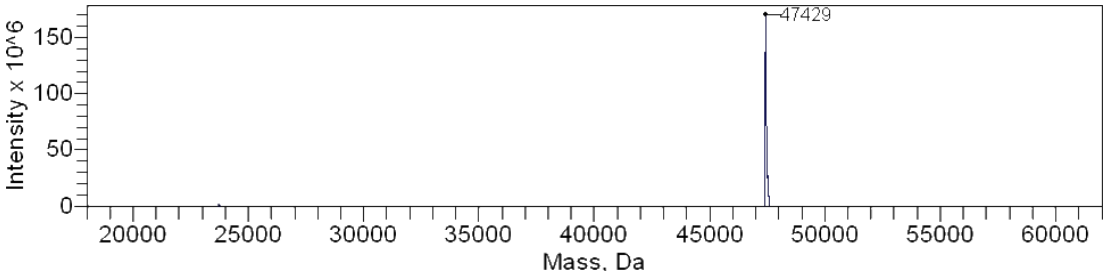
Reduced Rituximab Fab (eluted at 3.71 min; observed mass: 23040 (light chain); 24151 (heavy chain))



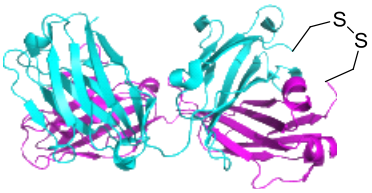
***N*-PEG succinimide bridged Rituximab Fab 166** (eluted at 3.69 min; expected mass: 47439; observed mass: 47429)

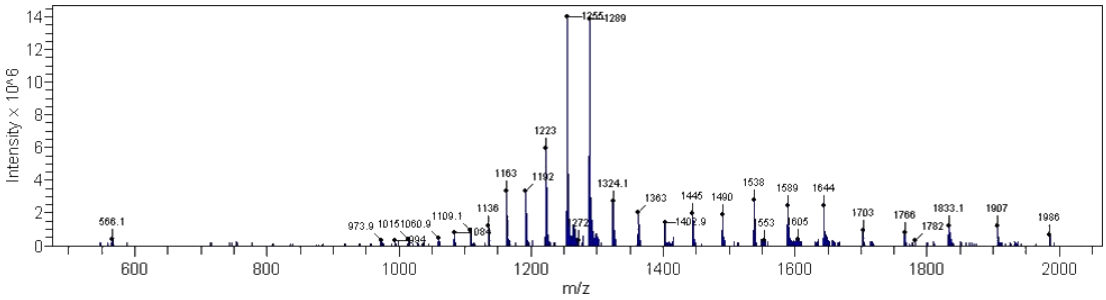


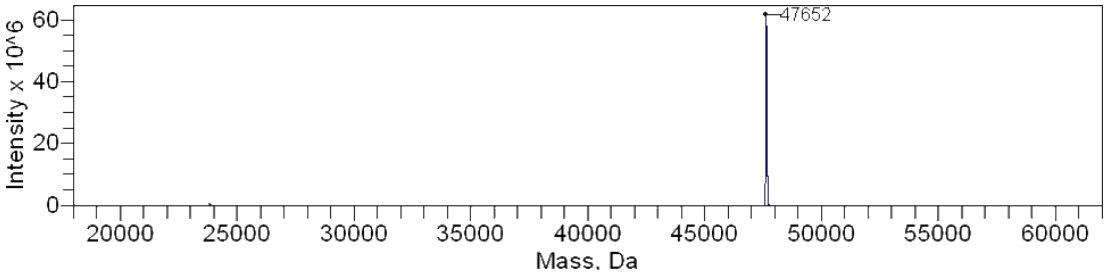




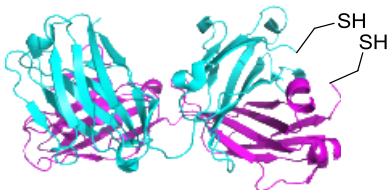
Unreacted Trastuzumab Fab 167 (eluted at 4.19 min; observed mass: 47652)

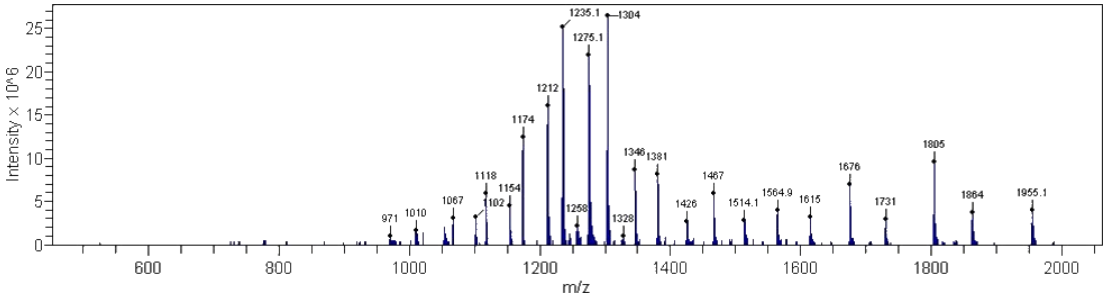


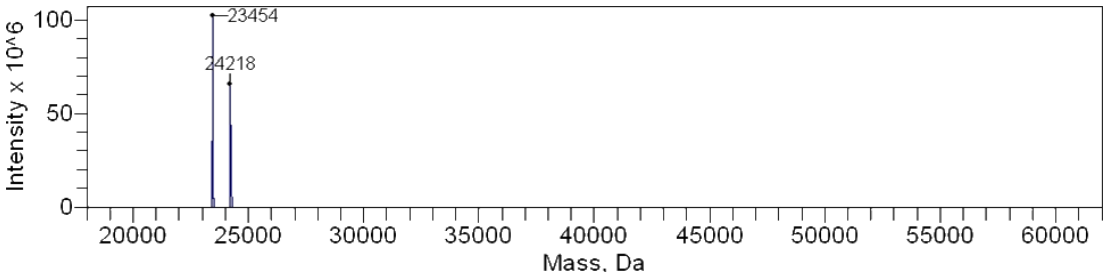




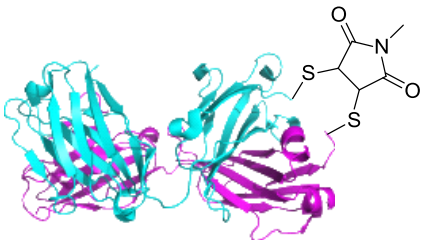
Reduced Trastuzumab Fab (eluted at 3.98 min; observed mass: 23454 (light chain); 24218 (heavy chain)

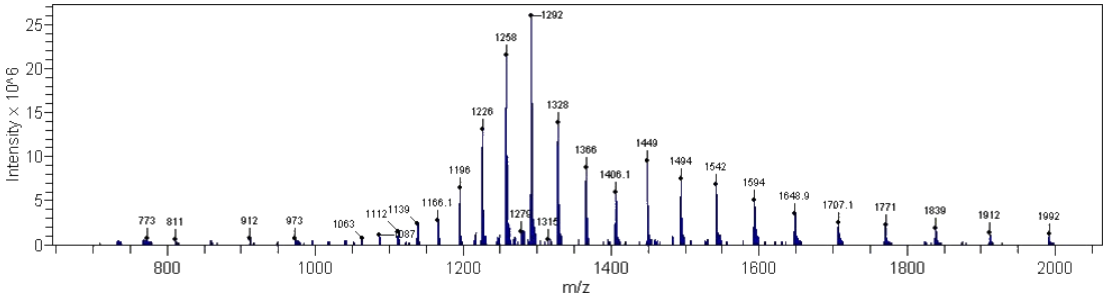


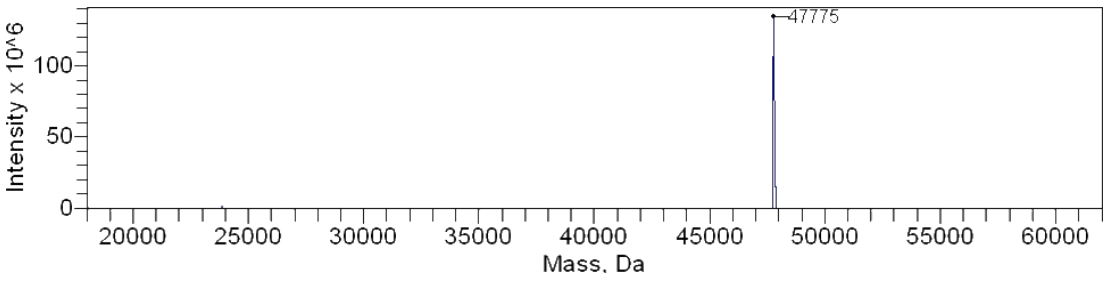




***N*-Methyl succinimide bridged Trastuzumab Fab 168** (eluted at 4.49 min; expected mass: 47767; observed mass: 47775)







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