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**ARTICLE TYPE**

# Aryloxymaleimides for cysteine modification, disulfide bridging and the dual functionalization of disulfide bonds

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**Tuning the properties of maleimide reagents holds significant promise in expanding the toolbox of available methods for bioconjugation. Herein we describe aryloxymaleimides which**
10 **represent 'next generation maleimides' of attenuated reactivity, and demonstrate their ability to enable new methods for protein modification at disulfide bonds.**

The maleimide motif **1** is a highly reactive conjugate acceptor and can be employed effectively in selective reactions with thiols,
15 such as cysteine residues in proteins.[1] As a result it is one of the most widely used functional groups in bioconjugation (Scheme 1). We have recently described bromomaleimides **3** as the parent members of a new class of reagents, which can be termed 'next generation maleimides' (NGMs). The incorporation of the
20 leaving group leads to a switch in the mechanism that occurs upon reaction with a thiol, from an addition reaction to an addition-elimination reaction. The result is the formation of a thiomaleimide product **4**, rather than thiosuccinimide **2**. Such next generation maleimide reagents enable new applications
25 including reversible cysteine modification,[2] the construction of triconjugates,[2b, 3] and the bridging of disulfide bonds.[2b, 4]
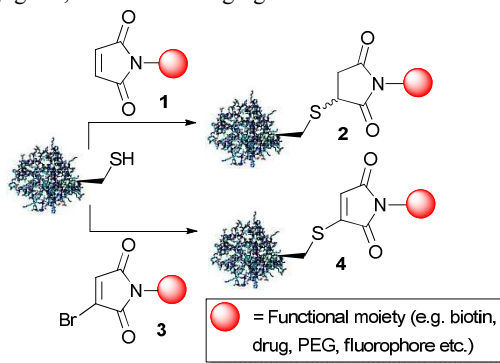


**Scheme 1.** Cysteine labelling of proteins using maleimide **1** and next generation maleimide (NGM) **3**.

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We envisaged that the choice of leaving group on the maleimide core could result in reagents with finely tuned properties. For example the reactivity could be controlled, in a manner similar to the use of iodo-, bromo- and chloroacetamides as varyingly
35 reactive reagents for selective cysteine modification.[5] In addition to guiding the reactivity, the choice of leaving group could also control other properties of the reagents, such as aqueous solubility. Herein we report on the aryloxymaleimides, as NGMs of attenuated reactivity, and demonstrate application of these
40 reagents in cysteine modification and in new modes of conjugation of disulfide bonds.

Having previously shown that thiophenol served as a suitable leaving group in dithiophenolmaleimide reagents,[4b] we postulated that phenol may do like-wise. Furthermore we considered that the
45 increased mesomeric contribution from the oxygen into the maleimide ring would lead to reagents of reduced electrophilicity. The synthesis of aryloxymaleimides **6-8** was carried out by simple addition of the corresponding phenol to *N*-methyl bromomaleimide **5** (Scheme 2).
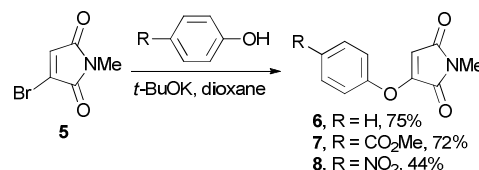


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**Scheme 2.** Synthesis of monoaryloxymaleimides **6-8**.

Addition of these reagents to a model protein, the single cysteine mutant (L111C) of the SH2 domain of the Grb2 adaptor
55 protein **9**,[2b] was monitored over 2 hours and compared to the use of bromomaleimide **5** (Fig. 1). All three aryloxymaleimides underwent the desired addition-elimination reaction to afford thiomaleimide conjugate **10**, at a reduced rate compared to bromomaleimide **5**.[2b] As expected, the incorporation of electron
60 withdrawing groups on the aryloxy ring (**7** and **8**) led to an acceleration in rate compared to the unsubstituted phenoxymaleimide **6**. It is notable that the aryloxymaleimides are still considerably more reactive than iodoacetamide, another commonly employed cysteine reactive reagent, which underwent
65 <5% reaction with this protein after 30 min under the same conditions.[2b] Furthermore the reaction could be accelerated by increasing the number of equivalents of phenoxymaleimide **6** to ten, leading to complete conversion to the product in just 10 min. These results demonstrate that phenoxymaleimides represent
70 cysteine modification reagents of attenuated reactivity, a feature we postulated could be useful in affording improved selectivity in certain bioconjugations.
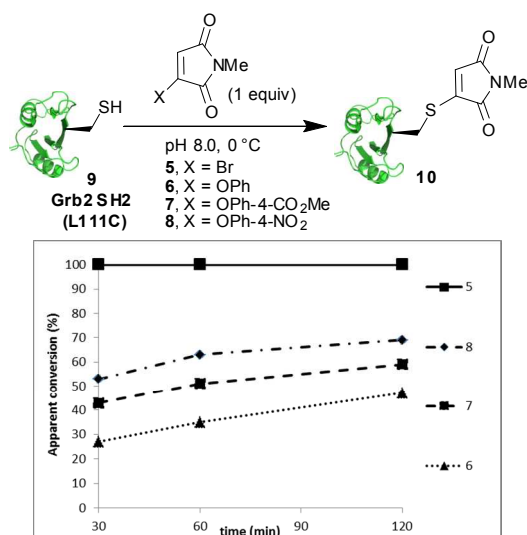
**Fig. 1.** Addition of aryloxymaleimides to Grb2 SH2 (L111C), with a graph of conversion over time measured by LCMS.

**Scheme 3.** Succinimide bridging of the disulfide bond in somatostatin.

A particular regioselectivity problem had become apparent in our studies on disulfide bridging for which we envisaged that the aryloxymaleimides could offer an ideal solution. We were interested in attempting to use bromomaleimide to bridge a disulfide bond in the peptide hormone somatostatin **11**, in a manner related to our work with dibromomaleimide.[2b, 4b] This would afford a saturated dithiosuccinimide **14** (presumed to be as a mixture of diastereomers), in contrast to the dithiomaleimides generated by dibromomaleimide bridging. Such dithiosuccinimides would be expected to have different properties to dithiomaleimides (e.g. reduced reactivity with thiols)[2b, 3b] and could serve as a complementary approach. However, addition of bromomaleimide **5** to reduced somatostatin **12** led to a mixture of the desired bridged adduct **14** and the *bis*-addition product **15** (Scheme 3). Thus addition of bromomaleimide to the second thiol in intermediate **13** was competing with the bridging reaction. What was required was an NGM analogue which underwent a slower reaction with cysteine, to ensure that the cyclisation of **13** would take place before the second maleimide addition. The aryloxymaleimides appeared to fit this specification. Indeed treatment of reduced somatostatin **12** with phenoxymaleimide **6** led after 1 h to quantitative conversion to the succinimide bridged somatostatin conjugate **14**. Increasing the amount of phenoxymaleimide to 10 equiv in this reaction still afforded none of the *bis*-labelled product **15**, demonstrating the extremely high selectivity for disulfide bridging. To prove that succinimide bridging had reached completion, product **14** was treated with 1 equiv of maleimide. No reaction was observed by LC-MS analysis after 5 min at room temperature, proving the absence of detectable amounts of free thiol.

We also found that, unlike bromomaleimides,[4b] aryloxymaleimides are resistant to cross-reactivity with the reducing agent TCEP and therefore that the succinimide bridging could be carried out *in situ*. The optimised *in situ* conditions involve addition of 1.5 equiv aryloxymaleimide to the peptide followed by addition of the equimolar amount of the reducing agent TCEP leading to quantitative conversion after 2 h. *In situ* protocols are valuable in disulfide bridging as they limit the amount of time the peptide or protein lacks its bridging functionality and thus reduces the propensity to aggregate or unfold.[4b, 6]
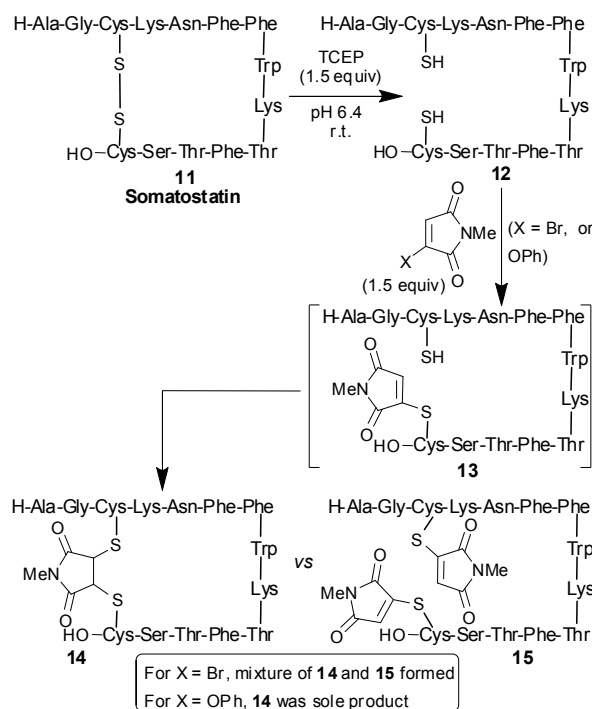
A selection of derivatised aryloxymaleimides (Fig. 2) were then synthesised (see SI Scheme 1) to explore the scope of this succinimide bridging. Notably a new, scalable synthesis of bromomaleimide was developed to allow convenient access to these molecules. A water soluble aryloxy motif was incorporated in **16** to investigate the possibility of organic solvent free labelling. A 'miniPEG' phenoxymaleimide **17** was developed as a model for PEGylation of proteins, and rhodamine phenoxymaleimide **18** for fluorescent labelling.
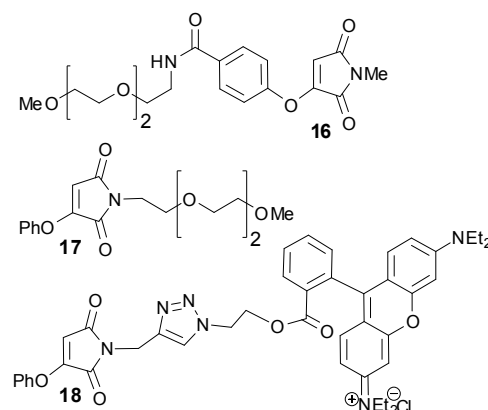
**Fig. 2**. Aryloxymaleimide derivatives.

The use of the water soluble aryloxymaleimide **16** successfully enabled the labelling of somatostatin under organic solvent free conditions. It is noteworthy that this is the first example of a maleimide reagent incorporating a water solubilising group which

is released upon conjugation, and further exemplifies the enhanced versatility of NGMs over traditional maleimides.

To demonstrate that the succinimide bridging was also applicable to larger proteins, chemical modification of an antibody fragment was investigated. A substantial portion of all biotechnology products under development are antibodies or antibody fragments,[7] and homogeneous conjugation technologies have a key role to play in realising the full potential in this area.[6, 8] Treatment of cystine stabilized anti-carcinoembryonic antigen single chain Fv fragment (anti-CEA ds-scFv) **19**[6] with *N*-miniPEG phenoxymaleimide **17** and benzeneselenol as the reducing agent[6] led to quantitative conversion to the conjugate **20** (Scheme 4). The same outcome was observe using *N*-methyl phenoxymaleimide **6** to afford conjugate **21**.
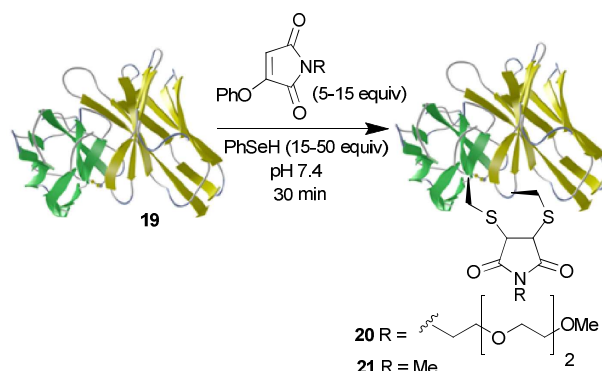


**Scheme 4.** Succinimide bridging of ds-scFv shMFE.

We have previously demonstrated that maleimide bridges are susceptible to thiol mediated cleavage at high concentration of thiols, such as the mM concentrations found in the cytoplasm of cells.[2b, 6] We wanted to test the stability of the succinimide bridge to thiols, to investigate if a different reactivity profile was demonstrated. Treatment of succinimide bridged scFv conjugate **21** with 100 equiv GSH (7 mM, pH 7.4) led to no reaction even after 48 h at room temperature. Thus the succinimide bridge in this scFv is completely resistant to thiol mediated cleavage in contrast to the maleimide bridge.

Returning to somatostatin however, it became apparent that this thiol stability is variable depending on the exact nature of the disulfide bridge. A comparison of maleimide and succinimide bridged somatostatin revealed that both were cleaved at similar rates by GSH under cytoplasm mimicking conditions to release the free peptide **12** (see SI Fig. 1). We propose that the mechanism for the thiol cleavage of the succinimide bridge starts with a retro-conjugate addition to generate acyclic thiomaleimide **13** (see Scheme 3), and thus **13** and **14** are in fact in equilibrium. Thiomaleimide **13** is then cleaved by GSH in a manner consistent with our previous results,[9] *via* a conjugate addition-elimination sequence. The difference in thiol stability of the somatostatin-succinimide **14** to the scFv-succinimide **20** we postulate to be attributable to the extent of intramolecular interactions that are present constraining these structures. In the case of somatostatin the formation of acyclic thiomaleimide intermediate **13** can be followed by a shift in conformation reducing the rate of rebridging and allowing a significant amount of this intermediate to form in solution. In contrast in the case of the scFv the

extensive interactions between the variable regions of the light and heavy chain hold the cysteines in close proximity, precluding the formation of significant amounts the acyclic thiomaleimide intermediate required for thiol mediated cleavage.

Notably employing *N*-phenyl phenoxymaleimide to bridge somatostatin led to the resultant conjugate which underwent rapid hydrolysis of the succinimide bridge (consistent with the equivalent *N*-phenyl maleimide bridge[4a], and see SI Fig. 2), affording a succinamic acid which was completely stable to thiols; this represents a general method of producing completely thiol stable conjugates.

Whilst the focus of this study was investigating the use of phenoxymaleimides in succinimide bridging, we realised that the reversibility of the bridging offered an interesting possibility for dual labelling of disulfide bonds. We envisaged that addition of a second electrophile could lead to interception of intermediate **13**, attaching a second tag. Dual labelling of a reduced disulfide bond is extremely difficult to achieve using traditional reagents as it relies on a significant difference in reactivity between the two cysteines.[10] Methods for the site-selective dual-labelling of peptides are widely sought, to allow controlled introduction of multiple functionalities.[11] For example, the incorporation of two different fluorophores allows FRET studies while the attachment of an *in vivo* stabilising group (*e.g.* a PEG) and a drug can prospectively enable therapeutic applications.

Thus treatment of reduced somatostatin **12** with phenoxymaleimide **6** (R[1]=Me) afforded the succinimide bridged somatostatin **14** which was then treated, without purification, with monobromomaleimide (R[2]=H) (Scheme 5). The reaction required warming to 37 °C for 2 h to ensure complete conversion to the dual modified peptide **22**. To confirm the formation of two cleavable thiomaleimides the conjugate was treated with 2-mercaptoethanol (100 equiv) to quantitatively convert to reduced somatostatin. To demonstrate that this approach could be employed to access dual functionalised peptides, we constructed miniPEG-fluorescein and rhodamine-fluorescein *bis*-conjugates (**23** and **24**). Notably other electrophiles can also be employed in the second modification step; for example maleimide was employed affording a dual conjugate in which one of the tags was cleavable by thiols and the other was not. Trypsin digest of **25** confirmed that the reaction led, as expected, to a mixture of the two possible regioisomers (see SI Fig. 3).
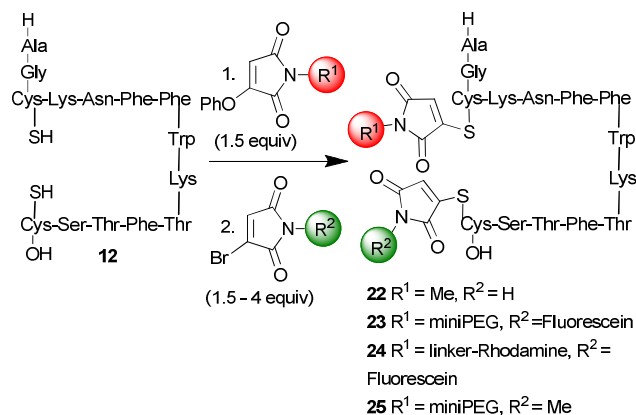
**Scheme 5.** Dual modification of somatostatin (conditions: 1. 1 h, r.t., pH 6.4; 2. 2 h, 37 °C, pH 6.4).

### Conclusions

In conclusion, by varying the leaving groups on a maleimide core, a range of next generation maleimides can be produced with tuneable properties. Here a selection of aryloxymaleimides have been demonstrated to serve as efficient cysteine labelling reagents of attenuated reactivity. This enables the aryloxymaleimides to be employed in succinimide bridging, offering a novel strategy for disulfide labelling. The reversibility of the succinimide bridge in the peptide also enabled a one-pot dual labelling strategy to be developed.

### Notes and references

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