Bromomaleimides: new reagents for the selective and reversible modification of cysteine†

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Bromomaleimides react rapidly and selectively with cysteine to afford thiomaleimides which can be cleaved with a phosphine to regenerate the cysteine or treated with a base to afford dehydroalanine.

Reagents that can selectively modify individual amino acids are of widespread use in the study of proteins. They allow us to mimic post-translational modification, to install tags such as fluorophores for visualisation or biotin for immobilisation and purification, to manipulate protein therapeutics, and broadly to enable bioconjugation.[1,2] Cysteine is often the most nucleophilic residue in a protein, and as such is generally regarded the easiest to modify in a selective manner.[1,3,4]

One of the most widely used reactive motifs for cysteine modification are maleimides.[3] Maleimides are extremely cysteine selective,[5,6] undergoing rapid conjugate additions *via* the thiolate of the cysteine residue.[7,8] Notably however maleimides react irreversibly and thus cannot be cleaved in subsequent steps to regenerate the free cysteine. Such reversibility could be extremely useful as it would allow the modification to be temporary. Applications for reversible cysteine modification include temporarily blocking an active cysteine residue,[9] protein purification,[10] quantitative proteomic analysis,[11] probing binding sites,[12] enabling structural studies[13] and in drug delivery.[14] Currently reversible cysteine modification can only be carried out using reagents that form disulfides such as methanethiosulfonates.[15] The disulfides can subsequently be cleaved with reagents such as DTT (dithiothreitol) or TCEP (tris(2-carboxyethyl)phosphine) to regenerate the cysteine. However cleavable disulfide reagents can be unstable, particularly with respect to disulfide scrambling.[11] We report herein on the bromomaleimides, the first of a new class of reagents for the selective and reversible modification of cysteine.

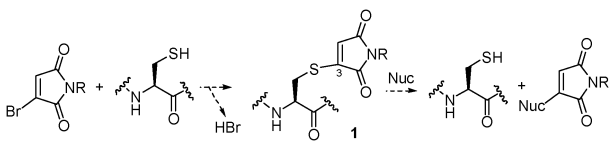
We postulated that incorporation of a leaving group on the maleimide double bond would enable an addition–elimination sequence to occur on reaction with cysteine. The product would be a thiomaleimide **1**, which retains a double bond, in contrast to the saturated succinimide product obtained from the addition of a thiol to maleimide. The thiomaleimide would have a new reactivity profile which could be exploited in subsequent steps. Of particular interest to us was the possibility that a nucleophile could add in another conjugate addition to C-3 and cleave the cysteine, reversing the modification (Scheme 1).

To the best of our knowledge the reaction of monohalomaleimides with thiols has never been reported before. We synthesised bromomaleimide **2** and bromo-*N*-methylmaleimide **3** by bromination of the corresponding maleimides and subsequent elimination of HBr.[16] We chose the commercially available *N*-Boc-Cys-OMe **4** as the cysteine model system on which to react the maleimides. Treatment of *N*-Boc-Cys-OMe **4** with bromomaleimide **2** resulted in a rapid (<1 minute) and extremely clean reaction. The product isolated was the expected thiomaleimide **5** in quantitative yield (Scheme 2). The same result was found with the *N*-methyl variant **3** with a slightly lower yield of 84% obtained. Sodium acetate was employed to neutralise the HBr, although this was not essential in the case of **5** which was stable to the highly acidic conditions. We also carried out the reaction of *N*-Boc-Cys-OMe **4** with bromomaleimide **2** in conditions suitable for a protein; 5% DMF–H_2O, again isolating **5** in 97% yield.

Intrigued by the rapid nature of the reaction we performed a competition experiment to reveal the relative reactivity of bromomaleimide compared to maleimide itself. *N*-Boc-Cys-OMe **4** was treated with a 1 : 1 mixture of bromomaleimide **2** and maleimide **7**. The result was a 7 : 3 mixture of the two products **5** and **8**, showing that bromomaleimide reacts faster with cysteine than maleimide (Scheme 3).

To check that this was the kinetic and not the thermodynamic outcome we carried out two experiments that confirmed the irreversible nature of the reactions. The addition of *N*-Boc-Cys-OMe **4** to bromomaleimide **2** followed after 10 min by maleimide **7** resulted exclusively in thiomaleimide **5**. In contrast addition of *N*-Boc-Cys-OMe to maleimide **7**, followed after 10 min by bromomaleimide **2** afforded exclusively succinimide **8**.

We then carried out experiments to model how selective these bromomaleimides would be for cysteine over other nucleophiles. A simple competition experiment between cysteine and propylamine was informative, with only the cysteine–maleimide adduct isolated in quantitative yield (Scheme 4). None of **9** was observed highlighting the excellent selectivity for thiol over amine nucleophiles. We also



Scheme 1 Proposed outcome for reaction of a cysteine residue with a bromomaleimide, and the reversal of the process by a nucleophile.

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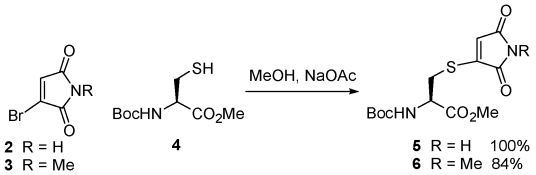
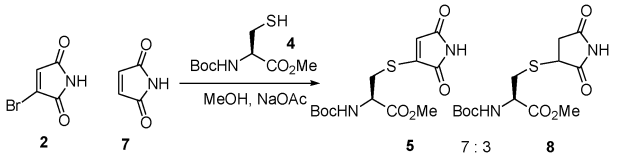


Scheme 2 Rapid reaction of bromomaleimides with *N*-Boc-Cys-OMe.



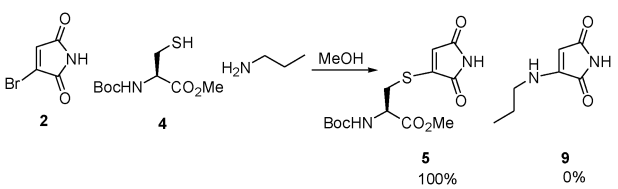
Scheme 3 Competition experiment between bromomaleimide and maleimide for *N*-Boc-Cys-OMe.

confirmed that bromomaleimide does not react with methionine. Treatment of bromomaleimide with *N*-Boc-Met-OMe resulted in no reaction after 2 days. This is in line with the proposed mechanism for cysteine addition to maleimides which involves the thiolate rather than the thiol.[7]

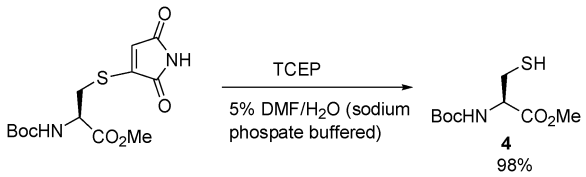
We then moved on to investigate the reactivity of these new thiomaleimide adducts with nucleophiles. Treatment of the thiomaleimide **5** with hexanethiol or another equivalent of *N*-Boc-Cys-OMe **4** resulted in no reaction after 2 h. This result indicates that thiomaleimides of this type are much less reactive to thiol conjugate additions than the bromomaleimides. This is likely due to a combination of electronics—the sulfur atom mesomerically releasing electron density into the maleimide ring—and sterics.

In contrast treatment of thiomaleimide **5** with water soluble phosphine TCEP in 5% DMF–H_2O (sodium phosphate buffered, pH 8) resulted in a clean reaction to afford *N*-Boc-Cys-OMe **4**, in 98% yield (Scheme 5). We used this buffer system as we wanted to employ conditions suitable for peptides and proteins. The $[\alpha]_D$ for **4** confirmed that no racemisation had taken place in this cleavage. With this convenient cleavage demonstrated we have illustrated the potential of bromomaleimide as a reversible modifying reagent for cysteine. In addition to applications in protein labelling bromomaleimides have potential applications in the temporary modification of thiols generally, for example as protecting groups in synthesis.

Another goal of our research has been to employ bromomaleimide to enable the conversion of cysteine to dehydroalanine (Dha). We envisaged this to be possible as the bromomaleimide is serving to convert the thiol into a good leaving group in the form of a thiomaleimide.



Scheme 4 Competition experiment between *N*-Boc-Cys-OMe and propylamine for bromomaleimide.

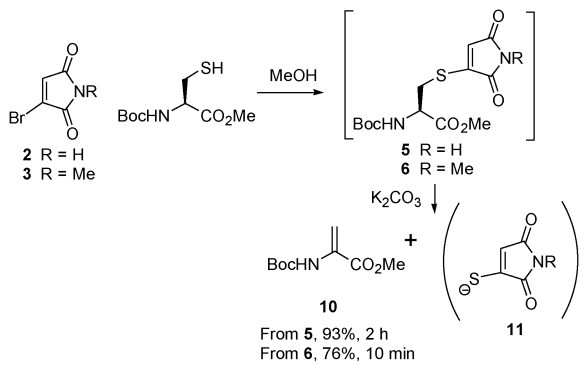


Scheme 5 TCEP (tris(2-carboxyethyl)phosphine) cleavage of the thiomaleimide.

Dha residues are found in a number of naturally occurring peptides, thought to provide rigidity to the peptide backbone and a reactive site for nucleophilic attack that is central to function.[17–20] Davis *et al.* have recently demonstrated the importance of the Cys to Dha conversion, reporting *O*-mesitylenesulfonylhydroxylamine (MSH) to carry it out.[21] The authors highlight the significance of avoiding unselective modification of other amino acid residues, such as methionine which is modified by peroxide based methods.[22] A potential drawback of MSH however is that it is explosive, and thus difficult to handle.[23]

In their model study Davis *et al.* use *N*-Boc-Cys-OMe **4**, treating it with MSH (10 equivalents) and K_2CO_3, to afford Dha **10**.[21] We were guided by these conditions and thus treated *N*-Boc-Cys-OMe **4** with bromomaleimide **2** (1 equivalent), followed by K_2CO_3. We were pleased to observe after 2 h the complete conversion to Dha **10**, isolable in 93% yield (Scheme 6).[24] The mixture turns yellow over the course of the reaction and it is proposed that this is due to the thiolate **11**, although this by-product has not been isolated. This Cys to Dha conversion can also be carried out with the *N*-methylmaleimide **3** to afford Dha **10** in 76% yield. Interestingly in this *N*-methyl example the reaction occurs in just 10 min which we suggest indicates that the thiomaleimide **5** is deprotonated under the reaction conditions hindering the rate of the elimination.

We are currently exploring this reaction further, however a few points should be noted at this stage. The reaction does not work well in water; while Dha **10** is observed to form by TLC analysis, it can only be obtained impure and in low yields (<5%). We believe the problems with water stem from the known susceptibility of maleimides to hydrolyse at pH > 8.[7] Currently therefore this method is limited to methanol as the solvent of choice. We believe we will be able to solve this in the future by developing base stable analogues to the bromomaleimides.



Scheme 6 Thiomaleimides are converted to Dha on treatment with base.

In conclusion we have demonstrated that bromomaleimides react rapidly and selectively with cysteine. In contrast to maleimides bromomaleimides can be employed for reversible cysteine modification as the thiomaleimide conjugate can be cleaved with TCEP. Furthermore these reagents can be employed for the conversion of cysteine to dehydroalanine. We envisage that variously functionalised maleimides incorporating a leaving group will provide an array of reagents for the reversible modification and protection of cysteine. We are currently investigating this further, along with the application of these maleimides to proteins, and will report in due course.

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24 It should be noted that Dha is prone to degradation, possibly by polymerisation, and maintaining the product in dilute organic solvent during the work-up is crucial.