# Enabling olefin metathesis on proteins: chemical methods for installation of *S*-allyl cysteine†

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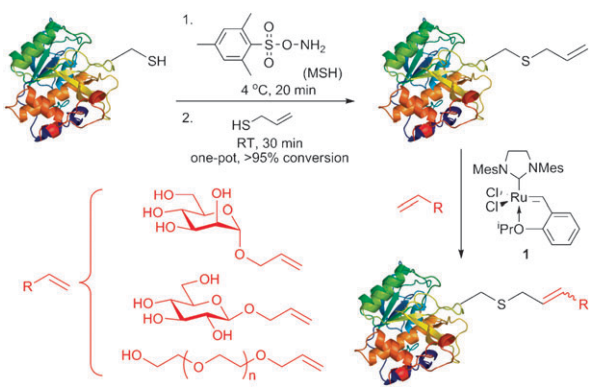
**Multiple, complementary methods are reported for the chemical conversion of cysteine to *S*-allyl cysteine on protein surfaces, a useful transformation for the exploration of olefin metathesis on proteins.**

Selective modification of biomolecules is a long-standing goal in chemical biology. In particular, precise chemical modification of proteins allows investigation of enzymatic and cellular activities and controlled alteration of macromolecular function.[1–4] As part of our continuing effort to expand the repertoire of selective methods for protein modification, we have set out to render transformations generally useful in organic synthesis as reliable methods for protein modification.[5,6] Among these reactions, olefin metathesis has emerged as a viable candidate for selective carbon–carbon bond formation on protein surfaces.[7,8]

Aqueous olefin metathesis has made strides in recent years,[9] facilitating ambitious applications of this reaction in chemical biology.[8,10] Complementing these advances is a maturing understanding of substrate reactivity in olefin metathesis and especially cross-metathesis.[11] For the purpose of protein modification this understanding is critical since it will guide the selection of the amino acid to be modified as well as its metathesis partner and any intervening linker. In our exploratory work in aqueous cross-metathesis, we discovered that allyl sulfides are privileged substrates and undergo rapid cross-metathesis in the presence of Hoveyda–Grubbs second generation catalyst **1**.[7,12] Taking advantage of the enhanced reactivity of allyl sulfides in olefin metathesis, we used the normally non-proteinogenic amino acid *S*-allyl cysteine (Sac) as the reactive handle for site-specific ligation of carbohydrates and poly(ethylene glycol) to protein surfaces. Sac was initially incorporated chemically into the model protein by the nucleophilic 1,4-addition of allyl thiol to dehydroalanine (Dha), which in turn was installed through the oxidative elimination of cysteine using *O*-mesitylenesulfonylhydroxylamine (MSH)[13] (Scheme 1).

While these first examples of cross-metathesis are very promising, the full scope of olefin metathesis for protein modification is yet to be determined. We considered that multiple, complementary routes to Sac would facilitate such investigations. We herein report alternative and selective chemical methods for the conversion of cysteine to Sac on proteins: direct allylation of cysteine with allyl chloride and allylation by an allyl selenenylsulfide rearrangement. We demonstrate that these methods are cysteine selective and provide metathesis-active Sac-containing proteins. Moreover, the electrophilic allylation and allylic selenenylsulfide rearrangement methods provide a single diastereomer of *S*-allyl cysteine, unlike the nucleophilic addition to dehydroalanine. Finally, the methods are mild, efficient, and easily accomplished without denaturing the protein.

The direct allylation of the amino acid cysteine[7,14] and cysteine-containing peptides[15,16] with allyl halides is well-known and the allylated products have been coupled to proteins through linkers[15] or by native chemical ligation.[17] However, to the best of our knowledge, direct allylation of cysteine on protein surfaces has not been reported. Perhaps the limited solubility of allyl halides in water or potential complications in selectivity have dissuaded such efforts. To investigate this transformation, we chose a single-cysteine mutant of subtilisin from *Bacillus lentus* (SBL-S156C) as a model protein. The single-cysteine construct simplifies reaction analysis and the protease activity of the enzyme can be assayed to ensure that the protein is not denatured during the modification. Allylation was attempted simply by treating a sample of SBL-S156C in pH 8.0 phosphate buffer with a solution of allyl chloride in DMF. We discovered that the total amount of DMF need not exceed 5% of the total volume of buffer to achieve complete homogeneity; this level of DMF is sufficiently low to be compatible with most proteins. Within 30 minutes at 37 °C full conversion to allylated protein **2** was observed (Scheme 2). Consumption of free thiol, and thus reaction at cysteine, was further verified by modified Ellman's assay (see ESI†).[13,18] The selective allylation of cysteine was observed even when a large excess of allyl chloride was used




**Scheme 1** Nucleophilic route to *S*-allyl cysteine.

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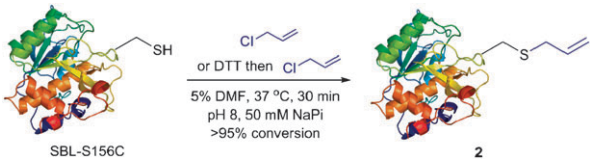
† Electronic supplementary information (ESI) available: Full experimental details, including $^{1}H$ and $^{13}C$ NMR spectra for all compounds and ESI-MS for all protein samples. See DOI: 10.1039/b908004j



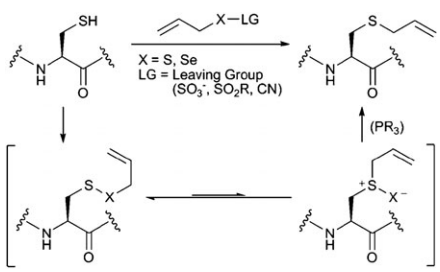

**Scheme 2** Electrophilic route to *S*-allyl cysteine.

(>1000 equivalents)—a testament to the unique nucleophilicity of cysteine. Furthermore, a protocol with pre-reduction of cysteine with dithiothreitol (DTT) was also demonstrated, a useful option for cysteines prone to oxidation. Finally, peptidase activity of the allylated protein was retained, as evidenced by liberation of *p*-nitroaniline upon treatment with the peptide suc-AAPF-pNA (see ESI†).[19]

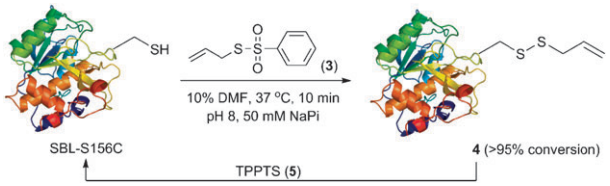
While the allylation of our model protein with allyl chloride is a selective and direct route to *S*-allyl cysteine, it is possible that, as with other alkylating reagents,[20] proteins containing strongly basic lysine or histidine residues may be allylated non-selectively. This selectivity problem may also be more pronounced when the target cysteine is hindered. It is therefore useful to have an allylation method that is *specific* for cysteine. The formation and dechalcogenative rearrangement of allyl disulfides and allyl selenenylsulfides is such a transformation (Scheme 3).

Seminal reports by Hoefle and Baldwin[21] and Sharpless and Lauer[22] on the rearrangement and reduction of allylic disulfides and allylic diselenides, respectively, inspired the Crich laboratory to use this chemistry as a cysteine-specific ligation strategy. Crich and co-workers have demonstrated the utility of this methodology in the specific allylation of cysteine in peptides up to 10 residues long in methanol or aqueous acetonitrile.[23–26] These transformations are efficient in aqueous media[23,25,26] since polar solvents stabilize the pericyclic transition state of the rate-determining rearrangement step.[27] The ease and specificity of disulfide and selenenylsulfide formation at cysteine and the ample precedence for the reductive rearrangement motivated us to explore the potential of this transformation on proteins as a route to *S*-allyl cysteine.

The allyl disulfide was investigated first. Treating SBL-S156C with allyl phenylthiosulfonate (**3**)[28] gave the expected allyl disulfide **4**. To promote the desired desulfurization, **4** was treated with the trisodium salt of triphenylphosphine-3,3′,3″-trisulfonic acid (TPPTS, **5**). TPPTS was chosen since it is a water-soluble derivative of triphenylphosphine, the phosphine used by Crich to promote desulfurization in




**Scheme 3** Dechalcogenative rearrangement of allyl disulfides and allyl selenenylsulfides.




**Scheme 4** Attempted desulfurization with TPPTS.

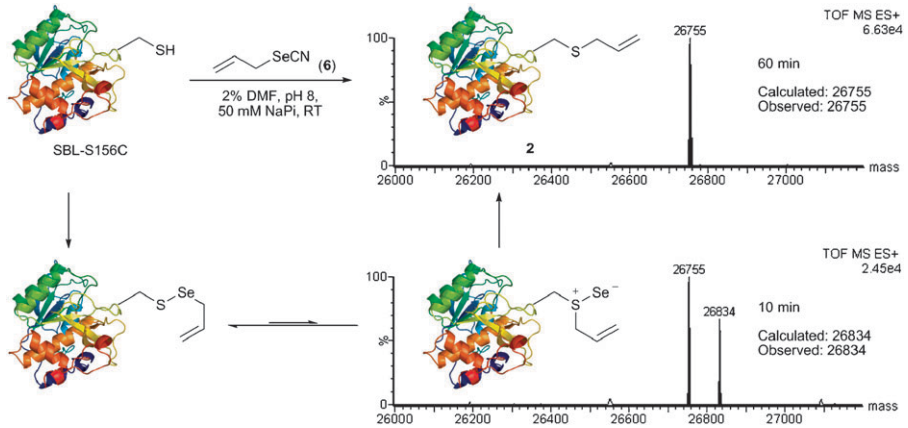
peptidic systems in less polar solvents.[23,25,26] Unfortunately only reduced product was observed (Scheme 4). Perhaps the unsubstituted allylic disulfide is too exposed and TPPTS too nucleophilic, resulting in direct attack on the disulfide rather than the thiosulfoxide product of rearrangement. While screening other phosphines may rescue this disulfide route to *S*-allyl cysteine, we instead turned to the analogous allyl selenenylsulfide system since loss of selenium is far more facile than the desulfurization attempted above and in many cases does not require phosphine,[24,25] an important feature for proteins with natural disulfides.

Allyl selenocyanate and *Se*-allyl selenosulfate are both suitable reagents for the conversion of cysteine to its *Se*-allyl selenenylsulfide.[24,25] We opted to use allyl selenocyanate (**6**) since previous reports indicated higher overall yields of allylated peptides using allylic selenocyanates than when the allylic selenosulfates were employed.[25] Gratifyingly, when SBL-S156C was treated with **6** at room temperature, smooth conversion to *S*-allyl cysteine was observed. LC-MS analysis revealed rapid formation of *Se*-allyl selenenylsulfide/*S*-allyl selenosulfide intermediates (MS calculated: 26 834; observed: 26 834) and spontaneous loss of selenium over the course of 1 h (MS calculated: 26 755; observed 26 755) (Scheme 5).

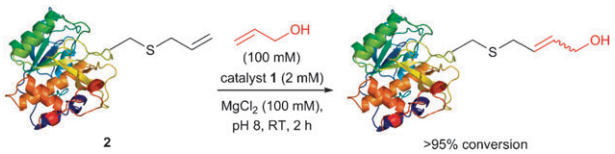
Full conversion was observed and the specificity for cysteine was verified by modified Ellman's assay (see ESI†).[13,18] Allylated protein **2** was an active peptidase, reflecting the mild nature of this allylation method. This is the first demonstration of the *Se*-allyl selenenylsulfide reductive rearrangement on proteins, a result that bodes well for the use of this ligation method in the synthesis of other lipidated proteins.

For our purpose, the overriding motivation for exploring methods for allylating cysteine is to provide flexible and efficient access to proteins suitable for olefin metathesis. As a demonstration of *S*-allyl cysteine reactivity in cross-metathesis and further corroboration of the formation of *S*-allyl cysteine by the transformations described above, the allylated proteins were used in a model cross-metathesis with allyl alcohol. Full conversion was observed after two hours at room temperature using catalyst **1** under previously optimized conditions (see ESI†).[7] As expected, no difference in cross-metathesis reactivity was observed between the sample obtained from allyl chloride and the sample obtained from allyl selenocyanate (Scheme 6).

In summary, we have investigated multiple chemical methods for the conversion of cysteine to *S*-allyl cysteine on proteins. The versatile chemistry of cysteine[29] allows mechanistic divergence in these strategies: the electrophilic allylation with allyl chloride and the reductive sigmatropic rearrangement of the *Se*-allyl selenenylsulfide of cysteine fully complement the nucleophilic addition of allyl thiol to dehydroalanine.




**Scheme 5** Cysteine-specific allylation by reductive rearrangement of *Se*-allyl selenenylsulfide.




**Scheme 6** Model cross-metathesis at *S*-allyl cysteine.

Together, these methods provide three modes of flexible entry to protein substrates suitable for olefin metathesis. This flexibility is important when adapting these reactions to a protein of interest. For instance, allyl thiol adds efficiently to dehydroalanine but might reduce natural disulfides; in this case allyl chloride and allyl selenocyanate would be more suitable. For a hindered cysteine, allyl chloride is more likely to allylate non-selectively; in this case allyl thiol addition to dehydroalanine (if there are no disulfides) and allylation with allyl selenocyanate should be the methods of choice. Regardless of the synthetic route, access to *S*-allyl cysteine-containing proteins is useful to elucidate the full scope of olefin metathesis as a protein conjugation method. The potential of olefin metathesis in bioconjugation[8,10,30] is driving our current research and progress to this end will be reported in due course.

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