Molecular BioSystems

This article was published as part of the

2008 Emerging Investigators Issue

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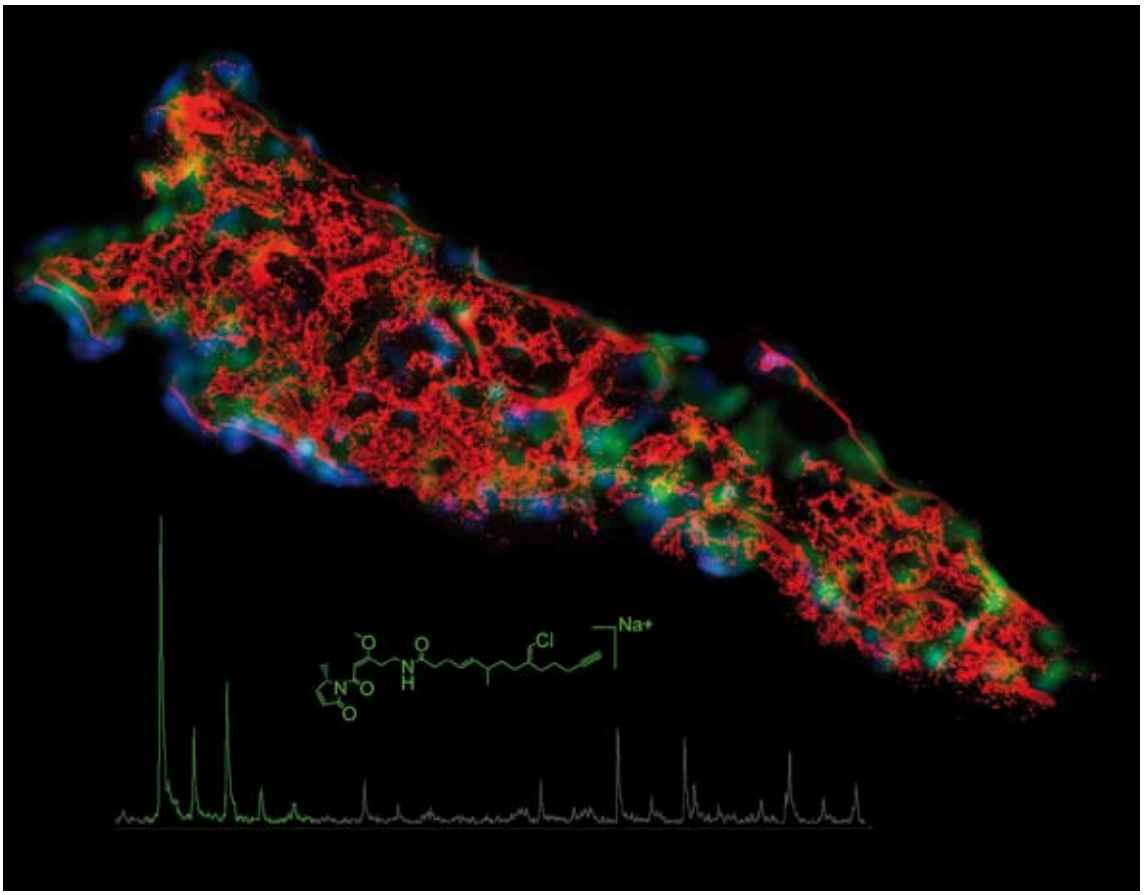

Chemical site-selective prenylation of proteins†‡§

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Received 7th February 2008, Accepted 25th April 2008
First published as an Advance Article on the web 2nd May 2008
DOI: 10.1039/b802199f

A direct thionation procedure allows conversion of allylic alcohols into the corresponding thiols, the products of which are immediately compatible with one-pot site-selective selenenyl sulfide mediated protein conjugation.

Post-translational modification (PTM) is a ubiquitous process in higher organisms contributing to higher organism complexity.[1,2] However, PTM proteins are typically produced bearing a heterogeneous mixture of PTMs and strategies for creating pure PTM proteins or their mimics are highly desirable.[1] Prenylation has been shown to be a major post-translational protein modification present on 0.5%–2% of all cellular proteins.[3] It is a lipid based modification involving the covalent attachment of isoprenyl anchors, either a C_{15} (farnesyl) or C_{20} (geranylgeranyl), to cysteine residues in consensus motifs such as the so-called CaaX box, CC or CXC (where 'a' is aliphatic amino acid and 'X' any amino acid) at the C-terminus of substrate proteins through a thioether linkage.[4–6] The attached prenyl is required for the correct function of the modified protein,[7] either as a mediator of membrane association or a determinant for specific protein–protein interactions.[8,9] Such prenylated proteins have been shown to play crucial roles in many cellular processes such as signal transduction,[10] intracellular trafficking[11,12] and cytoskeletal structure (*e.g.* maintaining retinal cytoarchitecture).[13] Moreover, inhibition of prenylation, in particular farnesylation, has recently become an attractive target for anti-cancer drug design, since oncogenic forms of Ras proteins require farnesylation for their ability to transform cells.[14] Reconstituted pathways in *Escherishia coli* allow the production of prenylated proteins[15] but the overall multi-step prenylation pathway is complex and does not always allow ready access to all target proteins and can show limited protein substrate flexibility.

Prescient work by Waldmann[16–18] demonstrated the power that chemistry might bring to bear on methods for the generation of probes and tools that could examine the effects of prenyl groups in proteins. Thus, attachment of prenylated peptides to truncated Ras variants allowed elegant probing of the role of lipdation in Ras-signalling.

Chemical methods for the direct attachment of lipids to peptides and proteins have also been explored but at present lack applicability or complete site-selectivity. Recently, Crich and co-workers have demonstrated an elegant allylic selenosulfide rearrangement as a method for modifying simple thiols.[19–21] This procedure allowed simple allylic systems to be ligated to thiols in protected cysteinyl peptides through the use of seleno-Bunte salts. However, when applied to prenyl-based systems the rearrangement gave substituted products in more moderate yields. An impressive example of protein prenylation employs palladium π-allyl complexes in a "solubility switching" strategy. Although not fully selective, this reaction allowed attachment of lipid modifications predominately to the phenolic oxygen of Y171 in chymotrypsin.[22]

We report here a chemical strategy that allows site-selective prenylation of a suitably tagged recombinant protein. This approach combines the introduction of a chemical tag into the protein backbone with control of the position by chemoselective modification of that tag.[2] As part of this strategy we chose a flexible linking method that mimics the natural cysteine-modified systems (Scheme 1). In this two-step approach a thiol is introduced into the protein backbone using site directed mutagenesis, and is subsequently targeted by a thiolselective prenylating reagent. A similar approach has previously enabled a variety of carbohydrate motifs to be conjugated to a selection of proteins in a controllable fashion.[23–28]

Thiol selective modification was based on the use of selenenyl sulfides. This method required good access to the corresponding prenyl based thiols and we investigated processes that would achieve this. Traditional methods typically allow the use of multi-step transformations from prenyl alcohols through activation of the hydroxyl as an ester or halogenation followed by displacement with sulfur nucleophiles (*e.g.* thioacetate,[29] thiourea[30]) and subsequent hydrolysis.[31] Not only did we seek a more direct route, but these methods can also suffer from poor regioselectivity.[32] We have recently used Lawesson's reagent (LR) for the direct chemo- and regioselective thionation of the reducing terminus of unprotected carbohydrates,[28] and reasoned that allylic alcohols would also act as substrates providing analogous S_N1-like reactivity.[33]

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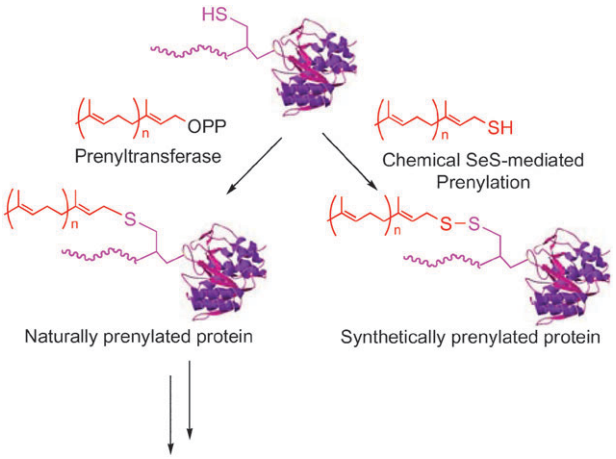
† This article is part of a *Molecular BioSystems* 'Emerging Investigators' issue highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces.

‡ Electronic supplementary information (ESI) available: Further experimental details. See DOI: 10.1039/b802199f

§ This paper is dedicated to the memory of Dr Joe Spencer.



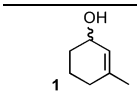
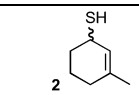
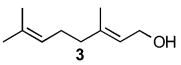
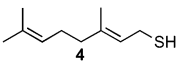
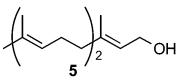
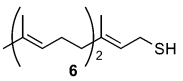
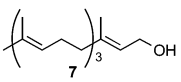
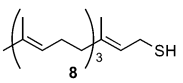
Scheme 1 Site-selective prenylation strategy: the use of selenenylsulfide (SeS) mediated prenylation allows the ready synthesis of posttranslationally-modified protein mimics.

Treatment of model allylic alcohol **1** with 0.6 equivalents of LR in anhydrous toluene at 80 °C gave the corresponding thiol **2** directly in 71% after 16 hours. With this pleasing result we shifted our attention to the biologically significant geraniol **3**, farnesol **5**, and geranylgeraniol **7**. Following the same protocol, it was possible to isolate the corresponding thiols in excellent yields (72–91%, Table 1). Reaction time is a key determinant of product composition with prolonged reaction times (*e.g.*, >20 h for **4**) giving rise to primary thiol (> 99%), whilst shorter reaction times (*e.g.*,<6 h for **4**) giving rise to a proportion of tertiary thiol (*e.g.*, 13 : 87, tertiary thiol : **4** after 3 h), suggesting thermodynamic control of regioselectivity, perhaps *via* [3,3]-sigmatropic equilibration of primary and tertiary arylphosphono- mono-, di or tri-thioate (–X–P(Ar)(=X)–S^-, where X = S or O) intermediates.

With a direct and efficient route to prenylated thiols established, their compatibility with protein conjugation was next investigated. A mutant (S156C) of subtilisin *Bacillus lentus* (SBL), containing a single free thiol tag, was pre-activated to its phenyl selenenyl sulfide intermediate, following exposure to phenylselenenyl bromide.[25] The next stage involved the addition of the prenylated thiols to the aqueous protein solution. In order to increase the solubility of the highly hydrophobic prenyl thiols a variety of co-solvents were screened (MeCN, THF and DMSO). Optimised conditions utilized 20% DMSO in aqueous buffer with periodic sonication (2 h). Analysis of the reaction was carried out using LC-MS and prenylated proteins were easily separated from starting materials (Fig. 1 and 2). It should be noted that conjugation yields were dependant on the size of the prenyl thiol used: geranyl >90%, farensyl >50%, and geranylgeranyl showed no conversion (and recovery only of pre-activated selenenated protein) due to the insolubility of geranylgeranyl thiol in aqueous buffer.

These synthetic prenylated proteins displayed functions that were dramatically modulated by modification. Hydrophobic interaction analysis (Fig. 3) as judged by octadecyl solid-support chromatographic retention showed significant retention shifts from single site selective modification with geranyl (SBL → SGL-geranyl: 25.3 min → 27.3 min) and farnesyl (SBL → SGL-farnesyl: 25.3 min → 28.4 min). Such clear

Table 1 Conversion of allylic alcohols to the corresponding thiols[a]

Substrate	Product	Yield (%)
		71[b]
		72[b]
		84[c]
		91[d]

[a] Reactions were carried with 0.6 equivalents of Lawesson's reagent in toluene at 80 °C under an atmosphere of argon. Reaction time is a key determinant of product composition with prolonged reaction times (*e.g.*, >20 h for **4**) giving rise to the primary thiol, whilst shorter reaction times (*e.g.* <6 h for **4**) give rise to a proportion of tertiary thiol. See ESI for details.‡ [b] >99% primary thiol. [c] >93% primary thiol. [d] >95% primary thiol.

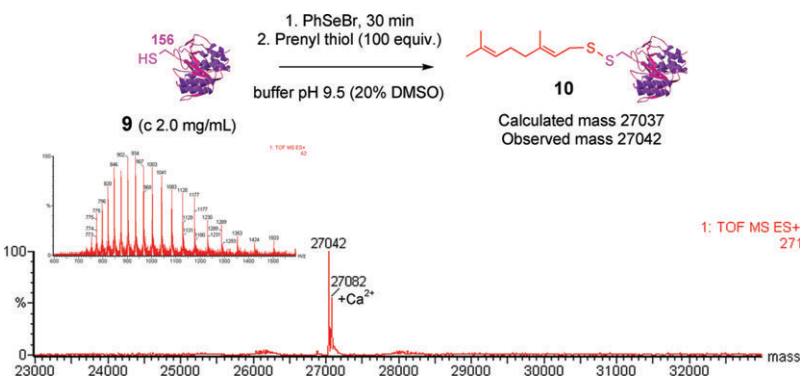


Fig. 1 ESI-MS spectrum of SBL-geranyl **10**.

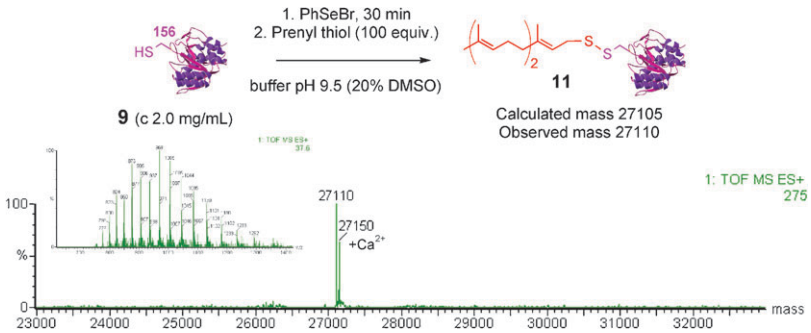


Fig. 2 ESI-MS spectrum of SBL-farnesyl **11**.

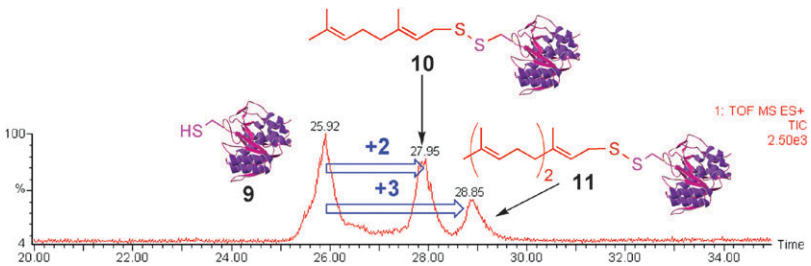


Fig. 3 Sample of unmodified (SBL **9**) and prenylated proteins (SBL-g **10** and SBL-f **11**) showing the dramatic effect upon hydrophobicity caused by prenyl conjugation.

hydrophobicity alterations from single site modification highlight not only the power of synthetic site-selective prenylation in protein function fine-tuning but may also suggest a similar role for prenylation observed in nature. The protein used here, SBL, also possesses naturally proteolytic activity that was retained (as judged by hydrolytic activity against succinyl-AAPF-*p*NA) after chemical prenylation, consistent with mild conditions that did not lead to denaturation. It should be noted that the protein we use here is not naturally prenylated and this suggests that principles of physical property tuning may be transferred even into unnatural situations. In addition, the disulfide link between prenyl and protein utilized here may be cleaved under extreme reducing conditions and we are currently exploring methods that will allow access to the natural thioether-linked analogues. A key question will be whether this form of site-selective chemical protein prenylation will reconstitute the properties of proteins that have been naturally prenylated; we are currently applying this approach to such systems and this work will be published in due course.

In conclusion, we have reported a direct and facile method for the preperation of allylic thiols from their parent alcohols using LR. Furthermore, the resulting thiols have been successfully conjugated in a site-selective manner to a protein. Previous methods have used either enzymatic methods[15] or chemical attachment of a peptide segment containing preinstalled prenylation.[16–18] To the best of our knowledge these results demonstrate one of the first examples of convergent, site-selective chemical protein prenylation directly at a tagged site of interest and we hope will prove complementary to these previous powerful methods. Further mimics of other post-translational modifications, and their compatibility with selenenyl sulfide methodology are currently being undertaken within this laboratory.

We gratefully aknowledge the support of the EPSRC (D.P.G), Glycoform (S.v.K.), the Fundação para a Ciência e a Tecnologia, Portugal (G.J.L.B.) and the Rhodes Trust (J.M.C.).

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33 Direct thionation of alcohols can be achieved selectively using Lawesson's reagent (LR) at centres capable of supporting S_N1-like substitutions. Thus, benzyl and anomeric hydroxyls [ref. 28] maybe directly converted to the corresponding thiols.