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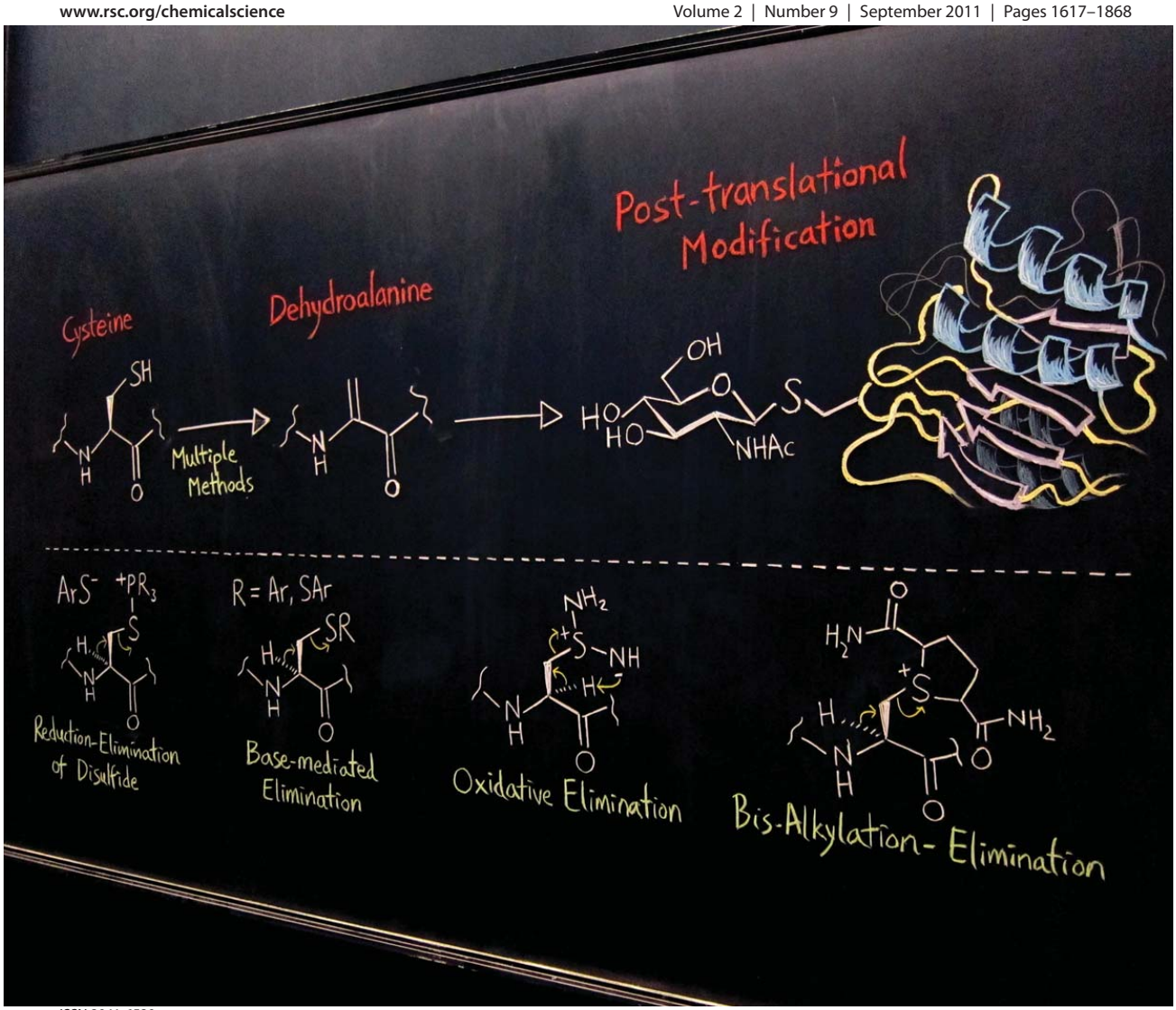

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EDGE ARTICLE

Methods for converting cysteine to dehydroalanine on peptides and proteins†

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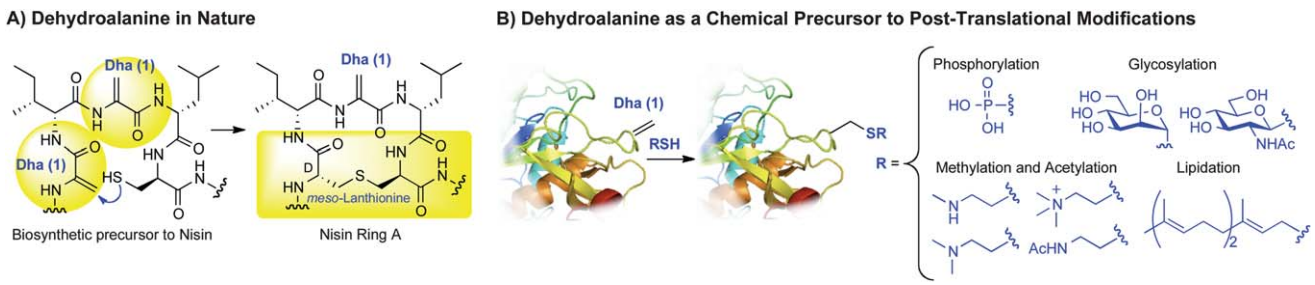
Dehydroalanine is a synthetic precursor to a wide array of protein modifications. We describe multiple methods for the chemical conversion of cysteine to dehydroalanine on peptides and proteins. The scope and limitations of these methods were investigated with attention paid to side reactions, scale, and aqueous- and bio-compatibility. The most general method investigated—a bis-alkylation–elimination of cysteine to dehydroalanine—was applied successfully to multiple proteins and enabled the site-selective synthesis of a glycosylated antibody.

Introduction

Dehydroalanine: a precursor to post-translational modifications

Dehydroalanine (Dha, **1**, Scheme 1) is an amino acid residue of both biological and synthetic interest. In nature, dehydroalanine is found in lanthionine-containing antibiotic peptides—lantibiotics—where it is formed by the enzymatic dehydration of serine.[1–3] Dehydroalanine imparts a conformational constraint on peptides[4] and is an electrophilic center for reactions with nucleophiles. Intramolecular additions to dehydroalanine occur naturally, as in the biosynthesis of lantibiotics such as Nisin (Scheme 1A).[1–3] Dehydroalanine also occurs naturally in proteins. For example, tyrosine is converted to dehydroalanine in thyroglobulin during thyroid hormone biosynthesis.[5,6]

Synthetically, dehydroalanine is a useful chemical precursor to a range of post-translational modifications (PTMs)[7,8] and their analogues by the conjugate addition of a thiol (Scheme 1B).[9–14] Protein modifications accessible *via* dehydroalanine comprise some of the most prevalent post-translational modifications in nature: phosphorylation,[15,16] glycosylation,[17–19] methylation,[20] acetylation,[21,22] and lipidation.[23] Though the addition of thiols to dehydroalanine can lead to epimeric products, this does not necessarily preclude their use in biology where one or both of the epimers can serve as enzyme substrates or enable binding of other



Scheme 1 Dehydroalanine is a precursor to modified peptides and proteins.

biomacromolecules. For instance, *S*-linked glycoproteins derived from Dha are acceptors for glycosylation by endoglycosidase A.[24] The sulfur-based methyl- and acetyl lysine analogs (Scheme 1B) have also been validated as lysine mimics in several biological contexts.[14,25,26] Phosphocysteine has been proposed as a mimic for phosphoserine[13] and is a natural protein modification,[27] as is farnesyl cysteine.[23] Both modifications are accessible through Dha (Scheme 1B).[13]

The importance of post-translational modifications in biomolecular function is well established,[7,8] but a detailed

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understanding of structure–function relationships of PTMs is often lacking. Studies of post-translationally modified proteins would therefore benefit from ready access to modified proteins such as those in Scheme 1B.[28] Native chemical ligation can supply post-translationally modified proteins and useful analogs with the atomic precision conferred by total chemical synthesis. However, this strategy is often technically challenging—especially when multiple ligations are required.[29] Amber codon suppression is another powerful technology that has provided proteins bearing post-translational modifications.[30–34] This method, however, requires the evolution of a unique aminoacyl-tRNA synthetase for each modified amino acid, and in some cases requires several additional chemical steps to access the final protein.[35–37] Moreover, for many residues bearing PTMs (*e.g.* trimethyllysine, phosphoserine, glycosylserines, and prenylated cysteines), no synthetases have been reported for their direct incorporation during translation. Dehydroalanine, in contrast, can be directly converted to several PTMs by an operationally simple conjugate addition of a thiol (Scheme 1B).

This route to modified proteins, however, is predicated on the efficient and reliable incorporation of dehydroalanine at the site of modification. Several chemical and biochemical methods for the incorporation of dehydroalanine into peptides and proteins have been described, yet each strategy has limitations. We discuss the merits and drawbacks of these methods below. This assessment helped frame our strategy for developing more general methods for the incorporation of dehydroalanine into protein substrates.

Previous reports for installing dehydroalanine in proteins

Site-selective incorporation of dehydroalanine into proteins has received the attention of chemists for nearly half a century. These efforts are summarized in Scheme 2. Initial reports from Koshland and co-workers described the conversion of the nucleophilic serine of chymotrypsin to dehydroalanine in an effort to investigate the role of the serine in catalysis.[38,39] After selective sulfonylation at the nucleophilic serine, base-mediated elimination provided dehydroalanine (Scheme 2A). Koshland *et al.* proposed that the electrophilic properties of dehydroalanine might enable "chemical mutations" by the conjugate addition of nucleophiles and demonstrated such additions to confirm dehydroalanine formation.[39]

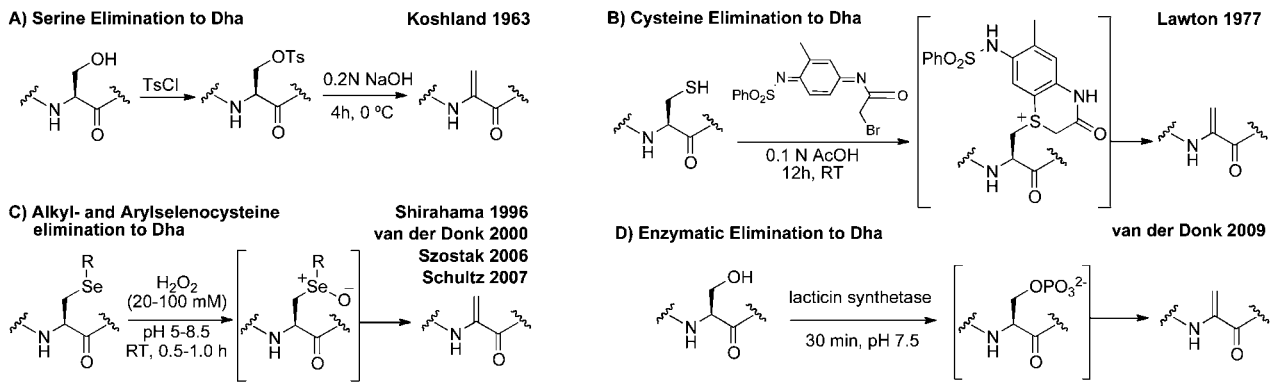
Koshland's pioneering work exploited the enhanced nucleophilicity of the active site serine in chymotrypsin. More general methods for incorporating Dha have utilized the nucleophilic properties of the cysteinyl thiol.[40] Holmes and Lawton, for instance, used the conversion of cysteine to dehydroalanine as a method for mapping cysteine residues since peptide-backbone hydrolysis occurs at dehydroalanine when heated (80 °C) under acidic conditions.[41] Cysteinyl residues were dialkylated and the resulting sulfonium salts eliminated to dehydroalanine after prolonged incubation in aqueous acetic acid (Scheme 2B).

Oxidative elimination of *S*-alkyl cysteinyl residues is another strategy for dehydroalanine synthesis; however, elimination of *S*-alkyl cysteinyl sulfoxides typically requires temperatures incompatible with protein substrates.[42] Oxidative elimination of phenylselenocysteine and related derivatives, in contrast, can be achieved *via* the selenoxide at room temperature and has provided access to dehydroalanine in peptides[43–45] and proteins[12,14] (Scheme 2C). Methods that exploit the promiscuity of enzymes in lantibiotic biosynthesis have also been reported for the conversion of serine to Dha (Scheme 2D).[46]

Despite this long-standing interest in dehydroalanine, the inherent limitations of the methods in Scheme 2 have precluded their wider use in peptide and protein modification. None of these methods enable general, chemo- and site-selective incorporation of dehydroalanine. For instance, base-induced elimination may require a pH incompatible with many substrates (Scheme 2A).[38,39] Lawton's method (Scheme 2B), requires a significant amount of organic solvent to dissolve the appropriate reagent which can cause protein precipitation.[41] Oxidative eliminations of alkyl-cysteines and alkyl-selenocysteines typically employ oxidants that react with other residues such as methionine (Scheme 2C).[14,45] Additionally, while lacticin synthetase (Scheme 2D) is relatively promiscuous, enzymatic routes to Dha are currently limited to peptides with specific leader sequences.[46]

Proposed methods for the conversion of cysteine to dehydroalanine

In this report, we disclose an assessment of methods for the conversion of cysteine to dehydroalanine. Cysteine is a convenient target for protein modification because of its strong nucleophilicity and ease of incorporation into proteins using standard biochemical techniques.[40] We consider and compare



Scheme 2 Representative methods for the incorporation of dehydroalanine into peptides and proteins.

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four complementary modes of reactivity for the conversion of cysteine to dehydroalanine: the reduction–elimination of cysteine disulfides, base-mediated elimination of cysteine disulfides and related derivatives, oxidative elimination of cysteine, and the bis-alkylation-elimination of cysteine (Scheme 3).

The reduction–elimination of cysteine disulfides (Scheme 3A) is designed on the basis of reports on the reaction of electron rich phosphines with disulfides. Phosphine attack upon disulfides is an endothermic process[47] and proceeds through a late transition state.[48,49] It should therefore proceed in such a way that the thiol with the lowest pK_a will be the leaving group. The logical consequence is that by making a mixed disulfide from an electron-withdrawing thiol and cysteine, attack on the sulfur of cysteine should be favored. The resulting phosphonium salt can then undergo elimination to give dehydroalanine. Harpp and Gleason observed similar eliminations from β-keto disulfides using electron rich phosphines,[50] Xian and co-workers observed the formation of dehydroalanine from cystine when treated with tris(dimethylamino)phosphine (HMPT),[51] and we have observed such eliminations from cysteine-carbohydrate disulfides using HMPT.[52]

While we anticipated that the intermediate thiophosphonium salt would eliminate more rapidly than the disulfide alone (Scheme 3A), we did not rule out the possibility of base-mediated elimination of the disulfide itself. Thus, after specific conversion to an electron-deficient disulfide, base-induced elimination is another route to dehydroalanine considered here (Scheme 3B).

A third method for the conversion of cysteine to Dha is the oxidative elimination of cysteine to dehydroalanine using *O*-mesitylenesulfonylhydroxylamine (MSH, **2**, Scheme 3C).[13] This reaction is nearly instantaneous between 0 °C and room temperature—a rate that compares favorably to the corresponding sulfoxide elimination of *S*-alkyl cysteinyl residues. In our initial report of this reaction, we applied this transformation to a single-cysteine mutant of a model protease, selectively converting cysteine to dehydroalanine.[13] While side reactions were not observed on this model protease, MSH is a reactive oxidizing and aminating reagent[53–55] and the full scope and limitations of this method are unreported.

A fourth method considered for the synthesis of Dha is the bis-alkylation–elimination of cysteine (Scheme 3D). This method is inspired in part by Holmes' and Lawton's method for cysteine mapping (Figure 2B).[41] We also noted that this transformation is observed in both murine and human metabolism of 1,4-dihalobutanes and Busulfan (1,4-bis(methansulfonyl)-butandiol) where the cysteinyl residue of glutathione was converted to dehydroalanine.[56–61] In these latter cases, the occurrence of these transformations under physiological conditions bodes well for application in biology as a synthetic tool for protein modification and *in vivo* chemistry.

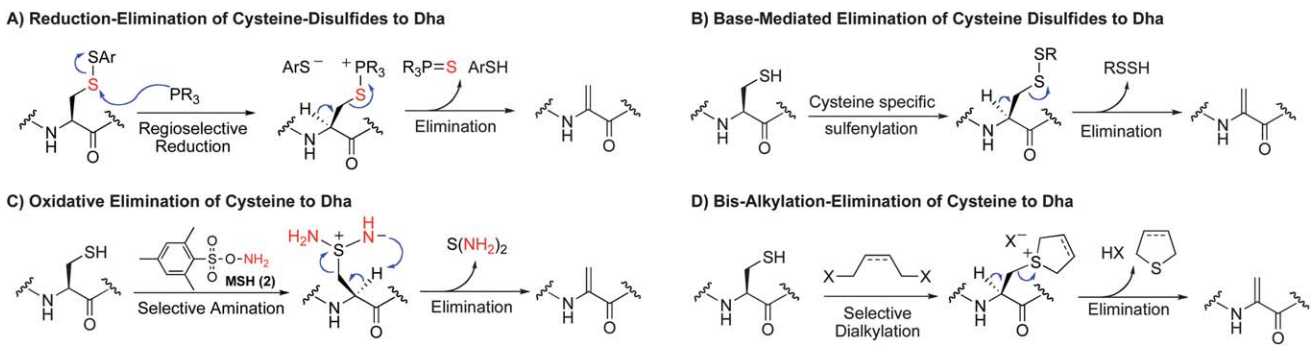
During these investigations, careful attention was paid to side reactions, substrate scope, synthetic limitations, and aqueous compatibility. After exploratory work on amino acid and peptide model systems, these methods were evaluated on several model proteins, including the protease subtilisin from *Bacillus lentus* and the single-domain antibody cAb-Lys3. We disclose the results of these studies here. Finally, the most general method for the conversion of cysteine to dehydroalanine was applied to several proteins and used in the site-selective synthesis of a glycosylated antibody.

Results and discussion

Conversion of cysteine to dehydroalanine on amino acid and peptide substrates

Conversion of cysteine disulfides to dehydroalanine under reducing conditions. We first tested the reduction–elimination route to Dha using disulfide **3** (Scheme 4). Disulfide **3** was synthesized using the methyl ester of Ellman's reagent (**4**).[62] The use of Ellman's reagent and its derivatives has several advantages. First, this reagent is specific for cysteine and the formation of the disulfide (*e.g.* **3**) is high yielding and rapid. Second, the *p*-nitroaryl thiol has a reduced pK_a and should make the arene thiolate the best leaving group in **3**. HMPT should therefore attack at the sulfur of cysteine since phosphine attack of disulfides proceeds through a late transition state (Scheme 4).[47–49] Third, Ellman's reagent itself is soluble in water and has long been used to specifically and quantitatively react with cysteine, especially in proteins.[62] Methyl ester **4** was used to ease handling and purification of **3**.

With **3** in hand, the reduction–elimination was tested. Upon addition of 2 equivalents of HMPT, the reaction mixture turned red, consistent with disulfide attack and release of the aryl thiolate. The major product of the reaction was the desired AcDhaOMe (**5**, 85%); phosphoramide **6** was also isolated in 46% yield. The formation of **6** is consistent with a mechanism in which the aryl thiolate leaving group attacks either the phosphine(thio) oxide (Scheme 4) or HMPT itself.[50] Importantly, AcCysOMe was not observed, indicating that HMPT attack at the arene



Scheme 3 Four complementary modes of elimination of cysteine to dehydroalanine.

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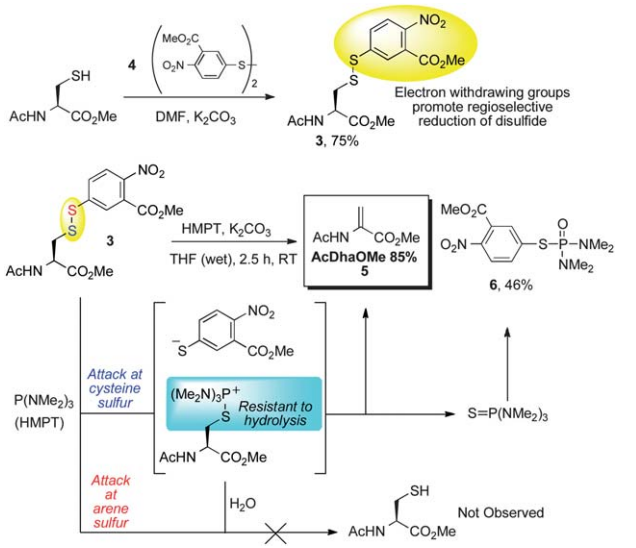
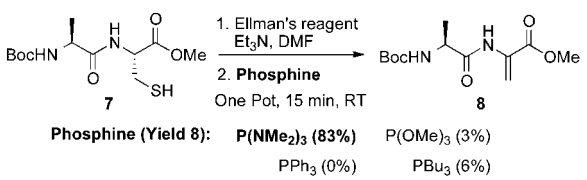


Scheme 4 Model reduction–elimination of cysteine disulfide.

sulfur of **3** is disfavored and that the hydrolysis of the cysteine phosphonium intermediate is slow relative to elimination (Scheme 4). It should be pointed out that carbonate is not a strong enough base to eliminate the disulfide directly (**3** was synthesized in the presence of carbonate); elimination therefore occurs after attack of the disulfide by HMPT.

With the desired reduction–elimination validated using model substrate **3**, we then investigated a one-pot procedure whereby cysteine is first treated with Ellman's reagent (as the free acid, rather than ester **4**) and then the resulting disulfide is reduced and eliminated using HMPT. DMF was used as the solvent and triethylamine as the base. Gratifyingly, Dha was generated efficiently from the dipeptide **7** in 83% yield (Scheme 5). While we anticipated that the electron-rich HMPT was the most suitable phosphine for the transformation in Schemes 4 and 5, we tested several other phosphorous nucleophiles since HMPT and the product of oxidation (HMPA) are toxic. The results of this screening are shown in Scheme 5. Unfortunately, though not unexpectedly, the use of other less electron-rich phosphines gave inferior yields and resulted mainly in reduction and regeneration of **7**.[63]

The successful use of disulfides as precursors to dehydroalanine is promising since they can easily be installed on proteins in a cysteine-specific manner.[40] The reduction–elimination strategy, however, is not the only method to convert cysteine-disulfides to dehydroalanine. In some cases, disulfides of cysteine can be eliminated directly to dehydroalanine.[64–68] We considered this method next.



Scheme 5 One-pot reduction–elimination of cysteine to dehydroalanine.

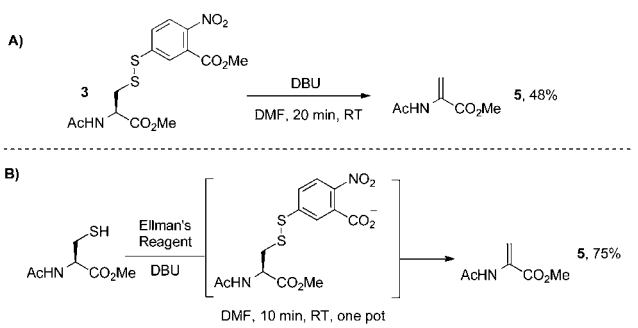
Conversion of cysteine disulfides to dehydroalanine under basic conditions. In exploratory work using Ellman's reagent in the reduction–elimination strategy, we found that DBU caused the direct elimination of disulfide **3** to AcDhaOMe **5** in moderate yield (Scheme 6A). If DBU is included in the mixture during reaction of a cysteinyl thiol with Ellman's reagent, Dha is formed in one-pot in 75% yield (Scheme 6B).

This DBU-mediated elimination is direct, simple, and scalable. However, it is known that disulfides can also be converted to the corresponding sulfinic acids in the presence of hydroxide, especially labile disulfides such as those derived from Ellman's reagent.[64] For this reason, the elimination of Ellman disulfides with base is likely limited to organic solvents and therefore not suitable for protein modification (*vide infra*). To resolve this issue, we considered other derivatives of cysteine that could undergo a similar elimination and better tolerate aqueous systems. In particular, we anticipated that Mukaiyama's reagent[69] was well-suited for this transformation given its high reactivity towards sulfur nucleophiles.[70]

Indeed, Mukaiyama's reagent reacted efficiently with cysteine. In DMF, the adduct of cysteine and Mukaiyama's reagent reacted to give dehydroalanine within 5 min in the presence of DBU (Scheme 7). The yield, however, was moderate and did not improve with extended reaction time. Treating AcCysOMe with DBU in the absence of Mukaiyama's reagent led to only trace AcDhaOMe (**5**).[63]

Unfortunately, while the use of Mukaiyama's reagent and base to convert cysteine to dehydroalanine is easy and scalable, it also requires a high pH (>10). Triethylamine is not basic enough to promote this elimination and application to protein substrates is likely limited to those tolerant of high pH (*vide infra*). We then turned to methods more likely to proceed at lower pH: oxidative elimination using MSH and the bis-alkylation–elimination strategy.

Oxidative elimination of cysteine to dehydroalanine using *O*-mesitylenesulfonylhydroxylamine (MSH). While it has been shown that MSH can selectively convert cysteine to dehydroalanine on protein substrates,[13] the potential side reactions of this aminating reagent have not been fully explored. In order to identify any such side products, we began by examining the reactivity of MSH with all proteinogenic amino acids with functionalized side chains. By identifying potential side reactions, we aimed to explore the scope and limitations of this method and identify experimental procedures that maximize selectivity. Partially protected variants of these residues were synthesized and



Scheme 6 Direct elimination of cysteine disulfides with DBU.

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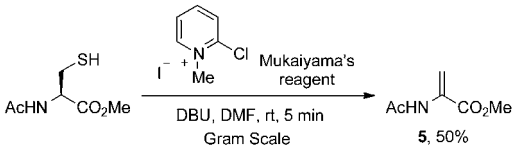


Scheme 7 Mukaiyama's reagent in the arylation and elimination of cysteine to dehydroalanine.

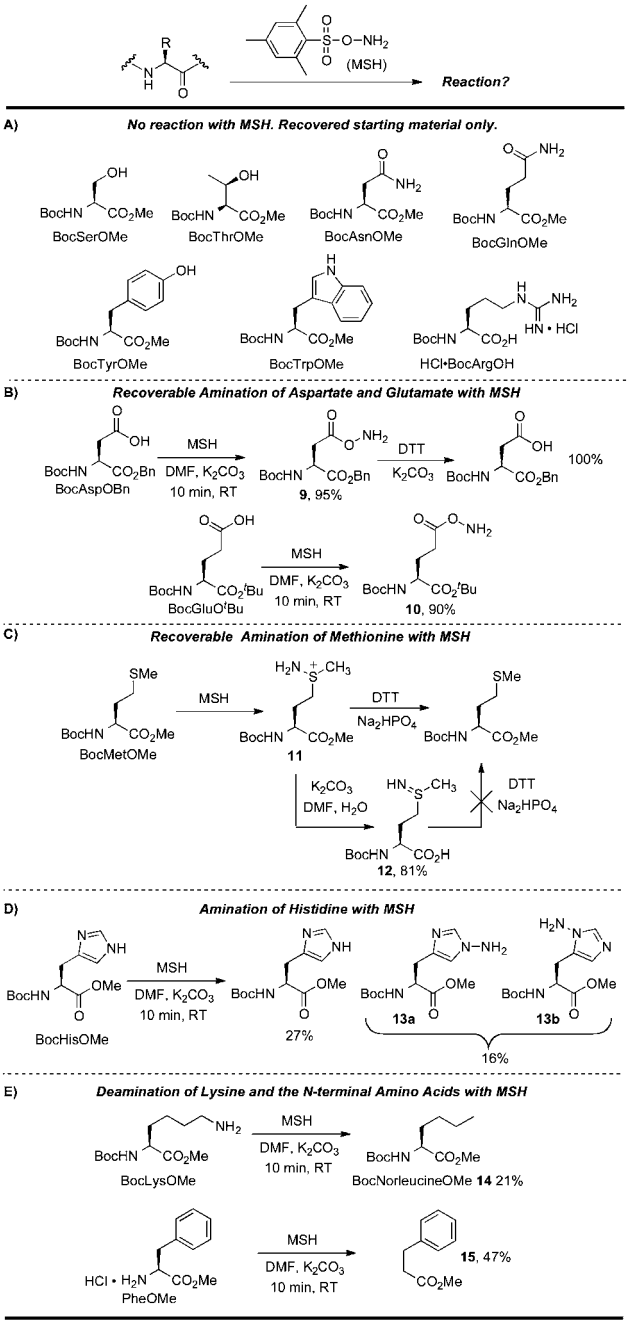
then treated with MSH, under conditions and times representative of those used for oxidative elimination of cysteine. The results of these experiments are compiled in Scheme 8.

No reaction was observed for the serine, threonine, asparagine, glutamine, tyrosine, and tryptophan derivatives in Scheme 8A under standard conditions. BocArgOH also did not react with MSH, likely because the basic guanidine side chain is protected in its protonated form. The free *C*-terminus of BocArgOH was also recovered without incident under these conditions. Carboxy groups can, however, be aminated by MSH under basic conditions, as seen in the reaction with aspartate and glutamate side chains. Efficient carboxy-amination was observed in these cases to provide **9** and **10** in high yield. While the rapid reaction of MSH with aspartate and glutamate revealed compatibility issues with these nucleophilic residues, the products of these aminations were easily reduced back to their respective carboxylic acid. For instance, treating **9** with dithiothreitol (DTT) led to rapid and quantitative reduction to BocAspOBn (Scheme 8B).

Methionine is also aminated by MSH, but the native thioether can again be regenerated using DTT. Therefore, this side reaction does not preclude use on peptides or proteins containing methionine because the resulting intermediate sulfiliminium salt (**11**) can be reduced back to the original amino acid residue (Scheme 8C).[71] Other reducing agents such as phosphines, zinc metal, sodium dithionite, and sodium ascorbate were not effective in this reduction.[63]

To clarify the role of the putative sulfiliminium **11** and discount other pathways for Met recovery, we examined other possible intermediates. For example, it was not clear whether DTT could reduce methionine sulfilimine **12** (as opposed to the sulfiliminium salt **11**). **12** was therefore isolated and reacted with DTT under basic conditions. It is notable that quite basic conditions (K_2CO_3, pH > 11) are required to generate **12** and it is unlikely that such an intermediate is formed during protein modification since a much lower pH is used in this reaction for protein modification (pH 8.0 or lower).[13] Even after 15 h, DTT did not reduce **12** to the thioether (Scheme 8C). This result suggests that the reducible intermediate is not sulfilimine **12** but another intermediate such as **11**. Finally, it is worth noting that double amination at sulfur was not observed in the synthesis of **12**, even in the presence of excess MSH.[63] This result suggests that for each methionine in a given substrate, it is likely that no more than a single amination will be observed at sulfur under conditions typical for protein modification. It is also notable that during these studies, no vinyl glycine or dehydrobutyrine was observed, indicating that elimination of **11** and **12** does not occur under these conditions.

Histidine and lysine also reacted with MSH (Scheme 8D and 8E). BocHisOMe gave a regioisomeric mixture of aminated products (**13a**, **13b**), consistent with previous studies that



Scheme 8 Reactions of MSH with functionalized amino acids.

describe the amination of nitrogen heterocycles with MSH and related aminating reagents.[53,72] Poor mass balance for this reaction suggests that some material is lost on workup, perhaps as the water-soluble imidazolium product of di-*N*-amination of the His sidechain. This proposal is consistent with a lower recovery with increasing equivalents of MSH.[63] Lysine gave a mixture of products after reaction with MSH. Deaminated lysine (norleucine derivative **14**) was the major product isolated from this mixture, albeit in relatively low yield (21%) (Scheme 8E). This result was initially counterintuitive: MSH, an aminating and oxidizing agent, effectively de-aminated and reduced lysine. This reaction, however, likely proceeds through the hydrazine—the

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product of amination at the ε-amino group—before further oxidation to the mono-substituted diimide and ultimate loss of nitrogen.[73] The course suggested for this reaction is depicted in Scheme 9 and is based on an analogous "hydrodeamination"[74] using hydroxylamine-*O*-sulfonic acid.[72]

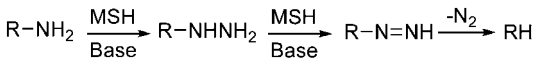
To investigate *N*-terminal deamination, PheOMe was treated with excess MSH. Deamination of the α-amino group was indeed observed and **15** was isolated in 47% yield (Scheme 8E). To further explore the limitations of an exposed *N*-terminus, model peptide **16** was synthesized and MSH was used to convert the cysteine to dehydroalanine (Scheme 10). While the desired peptide **17** could be isolated in useful yield, a significant side product was observed that had a mass that corresponded to loss of both H_2S and NH_2, consistent with concomitant deamination and Dha formation to give **18**.

From this assessment of MSH reactivity, it appears that the predominant side reactions occur at nucleophilic amino acid residues (Asp, Glu, Met, Lys, and His) and at the *N*-terminal amino group. For Asp, Glu, and Met, the product of amination can be converted back to the native side chain by reduction with DTT. Additionally, careful control of pH could minimize amination of lysine and histidine. MSH-mediated elimination of cysteine to dehydroalanine can proceed at a pH as low as 6 and lysine and histidine might be protected in their protonated forms.[13]

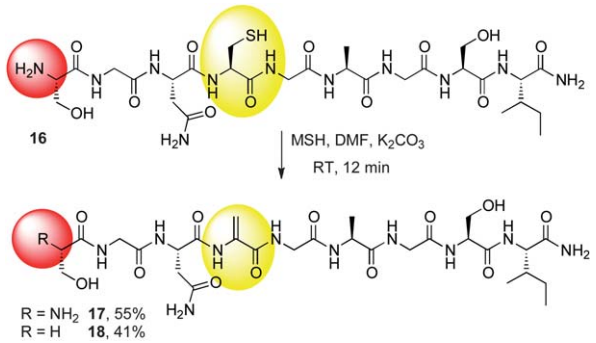
Therefore, while these side reactions can and do occur, they do not preclude selective reaction at cysteine. The selectivity that allowed successful conversion of cysteine to dehydroalanine on our model protein (subtilisin from *Bacillus lentus*) was likely due to a combination of factors such as the high nucleophilicity of cysteine, the pH, and differential accessibility of side chains.[13] These factors should be taken into consideration for use in peptide and protein modification. With a goal of avoiding these side-reactions entirely, we next pursued a potentially more selective transformation: the bis-alkylation–elimination of cysteine to dehydroalanine.

Bis-alkylation–elimination of cysteine to dehydroalanine. There were several parameters we considered in developing a general bis-alkylation–elimination procedure at cysteine (Scheme 3D): leaving group identity, the intermediacy of cyclic sulfoniums (including ring size), saturation levels, and water solubility. It was thought that optimizing these parameters would allow the best chance to execute this reaction at or near room temperature and at a mild pH. A model reaction in DMF between BocCysOMe and a variety of candidate electrophiles was our starting point (Scheme 11).

From the results in Scheme 11, several aspects of the bis-alkylation–elimination reaction are revealed. First, the 1,4-dibromo- and 1,4-diiodobutanes are the most efficient of the reagents screened to *S*-alkylate, cyclize, and eliminate. 1,4-Dichlorobutane is slow to alkylate and mostly unreacted BocCysOMe was recovered. Despite prior observations[56–61] that



Scheme 9 "Hydrodeamination" using MSH.

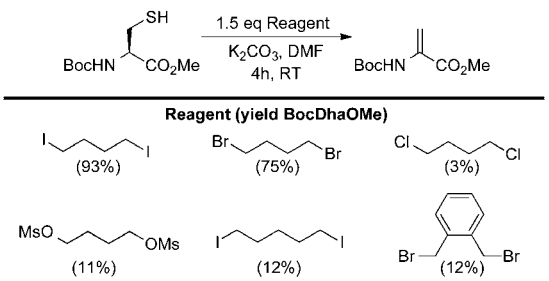


Scheme 10 *N*-terminal deamination using MSH on a model peptide.

had guided our reasoning (*vide supra*), Busulfan (1,4-bis(methansulfonyl)-butandiol) was also relatively inefficient under these reaction conditions, generating BocDhaOMe in only 11% yield. 1,5-Diiodopentane provided only 12% of the desired elimination product under the same conditions. This result suggested that formation of the six-member sulfonium ion is slower than the corresponding 5-membered ring formed with 1,4-diiodobutane or that the resulting cyclic sulfonium is less labile (or both). Finally, α,α′-dibromo-*o*-xylene generated only 12% Dha. In this example, the isolation of dimerized product **19** suggested that intermediate cyclization is slow (Scheme 12A). When a larger excess of α,α′-dibromo-*o*-xylene was used (5 equivalents) to minimize the formation of **19**, the major product was instead the alkylated, uncyclized benzyl bromide **20** which proved sufficiently stable to be isolated and characterized (Scheme 12B).[63] Heating **20** at 37 °C for 4 h in the presence of base led to modest yields of dehydroalanine (40%), providing further evidence for its intermediacy and relatively inefficient cyclization-elimination (Scheme 12C). Together, these results suggested that a saturated 1,4-dialkylating reagent would best enable efficient formation of the cyclic tetrahydrothiophenium intermediate, the immediate precursor to Dha.

The reconnaissance work depicted in Schemes 11 and 12 guided the design of water-soluble reagents with the requisite 1,4-dihalobutane moiety. The syntheses of these reagents (**21** and **22**) are outlined in Scheme 13.

Reagent **21** contains a hydrophilic tetraethyleneglycol unit tethered to a 1,4-diiodobutane core. Reagent **22** is a bisamide of the 1,4-dibromobutane core and was inspired in part by Kajihara's observations that diethyl *meso*-2,5-dibromoadipate converted cysteine to dehydroalanine on peptide substrates.[75] To



Scheme 11 Reagents for the bis-alkylation–elimination of cysteine to dehydroalanine.

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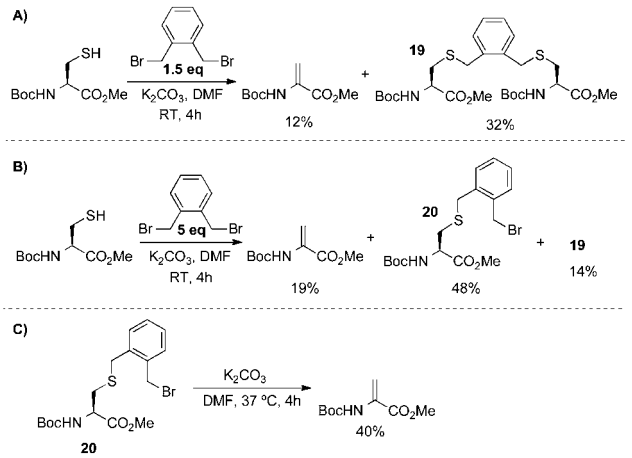


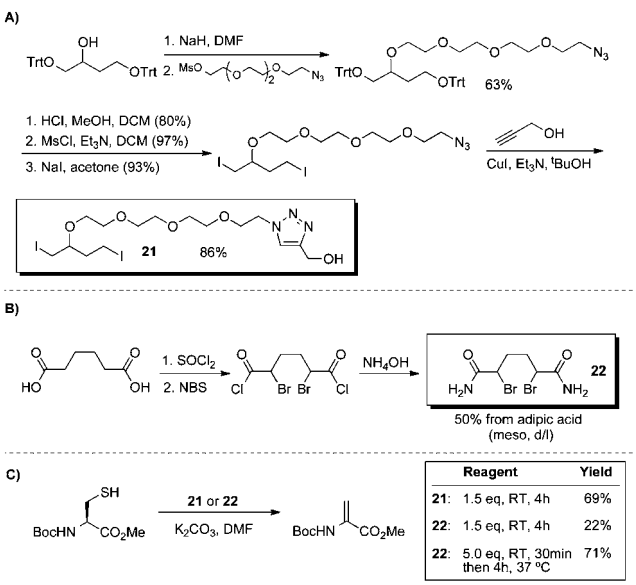
Scheme 12 Bis-alkylation–elimination with α,α′-dibromo-*o*-xylene.

compare **21** and **22** directly to the parent reagents in Scheme 11, the same model reaction was studied in DMF (Scheme 13C). Using di-iodide **21**, we obtained useful yields of Dha at room temperature. Bis-amide **22** initially afforded only a low yield of elimination product (22%), but with complete consumption of cysteine. It appeared that the cyclization was slow for **22**; when cysteine was first alkylated at room temperature and then incubated for 4 h at 37 °C, a 71% yield of Dha was obtained (Scheme 13).[76]

With a preliminary assessment of four modes of elimination of cysteine to dehydroalanine on model substrates in hand, their application to protein substrates was pursued next.

Conversion of cysteine to dehydroalanine on protein substrates

All protein reactions were monitored by LC-MS. The reaction conversions reported are calculated from the relative peak height of the deconvoluted mass spectrum. For a modification at a single site, we have previously shown that relative MS peak height correlates well with the relative amount of protein measured by independent methods.[77] For the substrates and chromatographic conditions employed, all protein material generally co-elutes in a single peak in the total ion chromatogram (TIC). In a typical analysis, the mass spectra for all protein material contained in this peak are combined and the resulting ion series is then deconvoluted using a maximum entropy algorithm. A representative analysis showing the TIC, combined ion series, and deconvoluted spectra can be found on page 55 of the supplementary information.†



Scheme 13 Synthesis of water-soluble 1,4-dihalobutane derivatives and their use in the conversion of cysteine to dehydroalanine.

Reduction–elimination of cysteine disulfides to dehydroalanine. In Scheme 4, it was demonstrated that regioselective attack at the cysteine-sulfur of Ellman-type disulfides and subsequent elimination of the phosphonium salt is a viable route to dehydroalanine. To investigate this reaction on a protein substrate, a single cysteine mutant of subtilisin from *Bacillus lentus* (SBL-S156C) was treated with Ellman's reagent to provide the requisite disulfide **23** (Scheme 14). When disulfide **23** was treated with HMPT at pH 8.0, rapid conversion to the phosphonium salt **24** was observed. No free cysteine was generated, indicating high selectivity for HMPT attack at the sulfur of cysteine in disulfide **23**. The phosphonium intermediate **24**, even after prolonged incubation, remained stable at pH 8.0. Subsequent pH adjustment (pH > 10) induced rapid elimination to Dha. Despite this successful conversion to Dha, the basic conditions required for this elimination do not meet the mild conditions necessary for a general method for the conversion of cysteine to dehydroalanine on protein substrates. This route was not pursued further.

Base-mediated elimination of cysteine disulfides and related derivatives to dehydroalanine. Intermediate **23** was also used to assess the feasibility of direct elimination of a cysteine disulfide to Dha. However, when **23** was treated with NaOH, no elimination was observed and the product had a mass consistent with direct attack of hydroxide on the labile disulfide, yielding, after air oxidation, the sulfinic acid **25**. Such formation of cysteine sulfinic acid from a disulfide has been observed in other protein substrates under alkaline conditions.[64]

The disulfide **23** derived from Ellman's reagent was not stable to nucleophilic attack by hydroxide and could not be directly eliminated to dehydroalanine. However, adduct **26**, formed from reaction of cysteine with Mukaiyama's reagent, can be eliminated under such conditions. Upon treatment with 1 M NaOH (pH ~11–12), rapid elimination to dehydroalanine was observed by LC-MS. The product protein did not react with Ellman's reagent, indicating that all cysteine was consumed in the reaction sequence. The presence of dehydroalanine was further corroborated by the conjugate addition of 2-mercaptoethanol.[63] The overall reaction sequence is depicted in Scheme 15.

While this 2-step, one-pot method for the conversion of cysteine to dehydroalanine is also restricted to substrates tolerant of high pH, it represents a selective and operationally simple method for installing dehydroalanine. Pursuing methods for dehydroalanine synthesis at a milder pH, we turned to oxidative and bis-alkylation–elimination.

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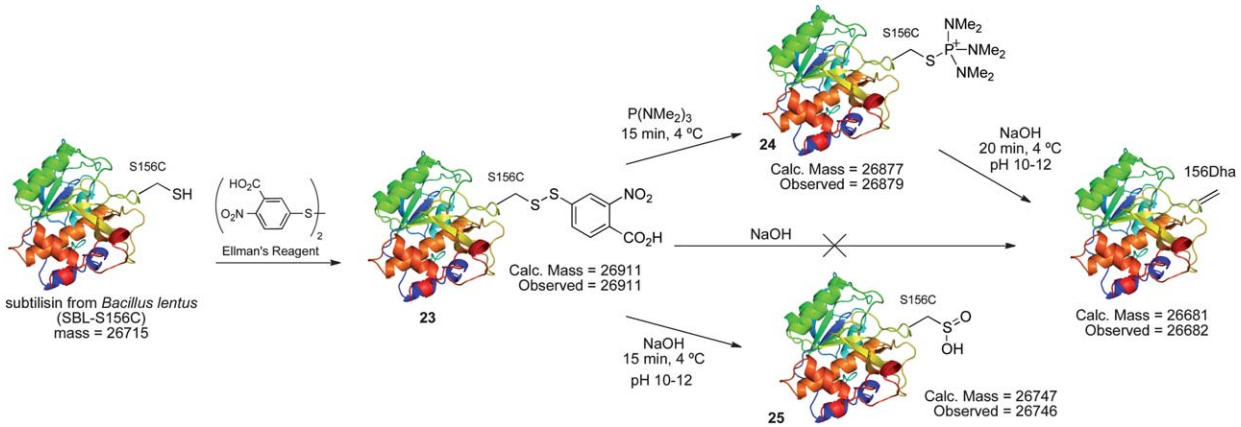


Scheme 14 Elimination of cysteine disulfides to Dha in protein model SBL.

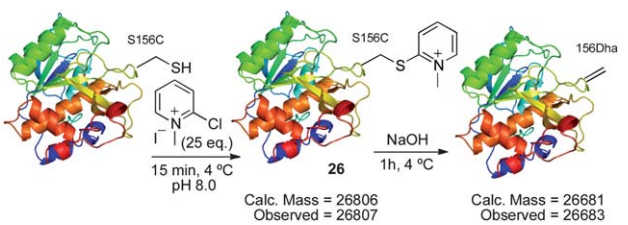
Oxidative elimination of cysteine to dehydroalanine using MSH. The oxidative elimination of cysteine to dehydroalanine with MSH has enabled access to a wide range of modified proteins (Scheme 1B and Scheme 16A).[13,24,78] However, this reaction was demonstrated on a single protein scaffold that bears a single cysteine on an exposed loop (subtilisin from *Bacillus lentus*, SBL-S156C). In an effort to extend this method to another protein, a single cysteine mutant of Np276 from *Nostoc punctiforme*, the ESI-MS clearly showed multiple products separated by increments of "NH_2" (16 Da) (Scheme 16B).[24,63] In light of the results in Scheme 8, this non-selective reaction is perhaps unsurprising. The cysteine residue at position 61 is hindered[24] and, unlike SBL-S156C, this Np276 mutant is His-tagged (a potential site of non-selective amination, Scheme 8D). Unfortunately, lowering the pH to 6.0 did not result in selective reaction.[24,63] We saw this result as an opportunity to generalize the conversion of cysteine to dehydroalanine on a more challenging substrate using more selective chemistry. Accordingly, we turned to the bis-alkylation–elimination method and an assessment of **21** and **22** in the conversion of cysteine to dehydroalanine on protein substrates.

Bis-alkylation–elimination of cysteine to dehydroalanine. Di-iodide **21** and di-bromide **22** were first evaluated on SBL-S156C to gauge their efficiency in the conversion of cysteine to dehydroalanine on protein substrates. When SBL-S156C was treated with **21** and incubated for 1 h at 37 °C at pH 8.0, elimination was observed by LC-MS.[63] Reaction at cysteine was confirmed by Ellman's assay and the presence of dehydroalanine was corroborated by the addition of 2-mercaptoethanol (Scheme 17A).

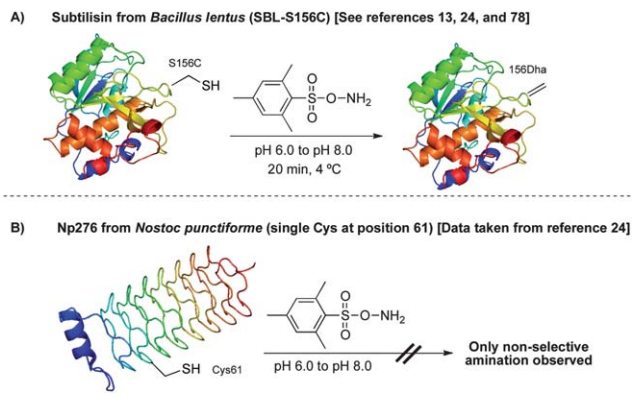
A similar reactivity was observed with **22** on SBL-S156C. After 30 min at room temperature, the alkylated uncyclized intermediate was detected by LC-MS (Scheme 17B). This outcome was consistent with the model reactions in Scheme 13C. To induce cyclization and elimination, the reaction was simply incubated for one hour at 37 °C. Full conversion to dehydroalanine was observed (Scheme 17B). Conversion of cysteine to dehydroalanine was confirmed by Ellman's assay and addition of 2-mercaptoethanol.[63]

The reaction of di-bromide **22** with the single cysteine mutant of Np276 was examined next (Scheme 17C). Using **22**, the selective conversion of cysteine to dehydroalanine was indeed possible, overcoming the non-selective amination observed with MSH. Reaction at cysteine was once again verified by Ellman's assay and the formation of dehydroalanine was demonstrated by the addition of various thiols. For instance, the dehydroalanine residue was subsequently converted to *N*-acetyl-glucosamine cysteine (GlcNAc cysteine).[24,63] The synthesis of this *S*-linked glycoprotein was critical in our concurrent investigation of endoglycosidase A in glycoprotein synthesis.[24]

As a more demanding test for reagent **22**, a mutant of the camel single-domain antibody cAb-Lys3 was used as a substrate (cAb-Lys3-A104C, Scheme 17D).[79,80] This antibody mutant contains a single reactive cysteine at position 104 and two disulfide bonds located in the core of the antibody. The correct folding of the A104C mutant was inferred by an enzyme-linked immunosorbent assay (ELISA) that showed binding to hen egg



Scheme 15 Elimination of cysteine to dehydroalanine after reaction with Mukaiyama's reagent.



Scheme 16 MSH in the oxidative elimination of cysteine to dehydroalanine on protein substrates.

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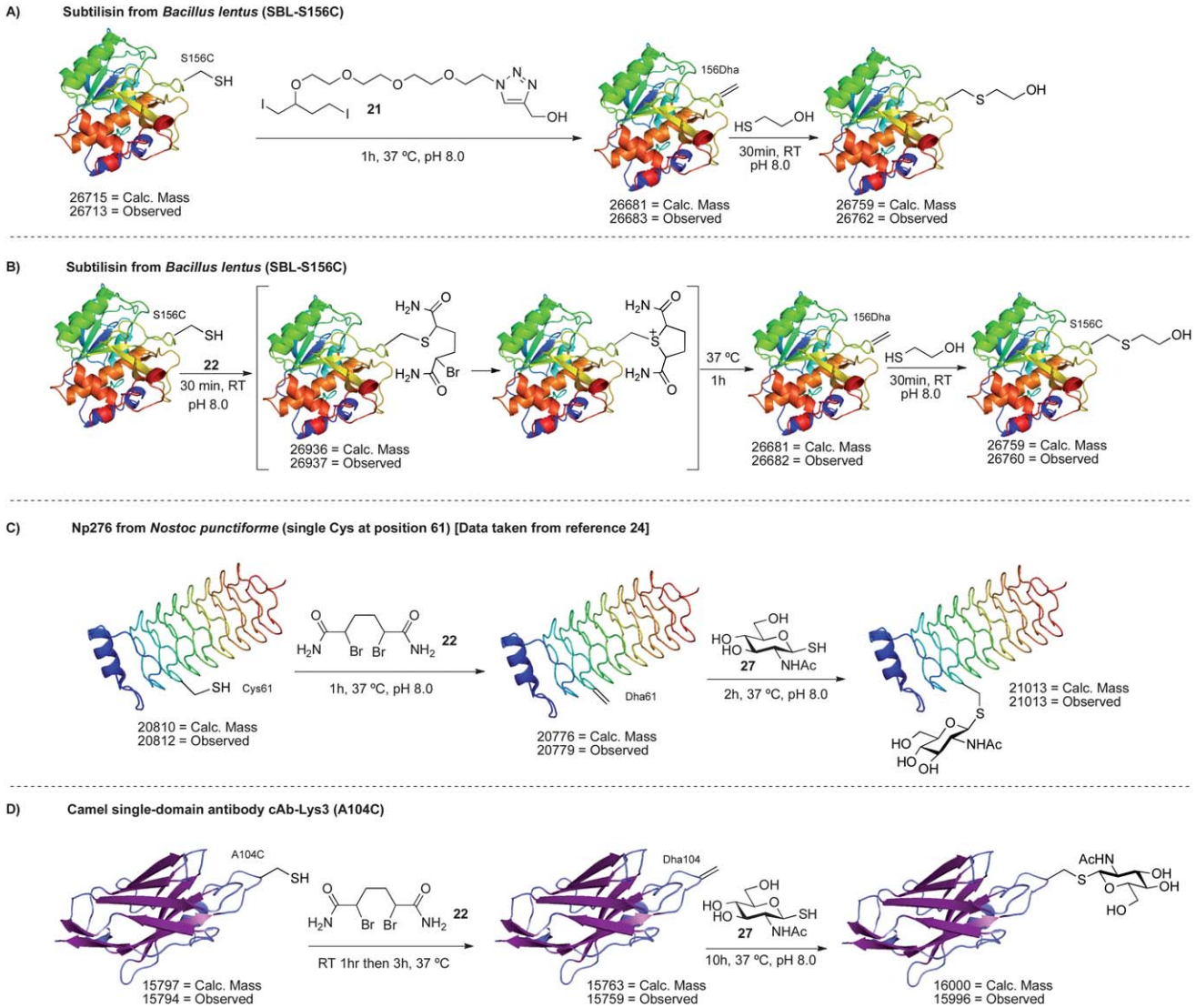

Scheme 17 Bis-alkylation–elimination of cysteine to dehydroalanine on protein surfaces.

white lysozyme, the antigen to which cAb-Lys3 was raised. Circular dichroism (CD) spectroscopy also showed a secondary structure comparable to the wild type antibody.[63] Upon reaction of the A104C mutant with **22**, efficient conversion to dehydroalanine was observed by LC-MS (Scheme 17D). The protein product did not react with Ellman's reagent, demonstrating all free cysteine was consumed in the reaction, and the formation of Dha was corroborated by the addition of 2-mercaptoethanol.[63] In control experiments, it was shown that the wild type antibody did not react with **22**, Ellman's reagent, or 2-mercaptoethanol.[63] These results indicate that the reaction observed for the mutant antibody occurs at cysteine 104. It is notable that in this example, no organic solvent was used and **22** was simply added as a solid—an important feature for fragile proteins such as antibodies that may not tolerate organic solvent. It should also be highlighted that this reaction sequence is a rare example of regioselective cysteine modification[81–83] and demonstrates that the incorporation and modification of a single cysteine 'tag' is possible in proteins that contain multiple natural cysteine residues.[84,85] Of course, such selectivity is less likely when the protein contains multiple reduced cysteines, rather than disulfides, but regioselective modification can still be achieved if these cysteine residues have different solvent accessibility.[81]

Finally, the dehydroalanine on cAb-Lys3 was converted to a GlcNAc cysteine residue by the addition of the glycosyl thiol of *N*-acetylglucosamine (**27**). *S*-Linked glycopeptides are found naturally[86–88] and we propose their use as analogs of the more commonly found *O*-linked glycoproteins.[89] Moreover, antibody glycosylation often improves their pharmacokinetic and biophysical properties.[90,91] Not surprisingly, much attention has been devoted to the synthesis of homogeneously glycosylated antibodies in recent years.[92–96] We note here that conjugate addition to dehydroalanine is one of the few methods for the site-specific synthesis of *S*-linked glycoproteins and the results in Scheme 17 attest to the facility of **21** and **22** in accessing these macromolecules.[97,98]

Conclusions

We have disclosed an assessment of multiple, complementary methods for the conversion of cysteine to dehydroalanine—

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a useful 'tag' in the 'tag-and-modify' approach to protein modification.[84,85] Side reactions, selectivity issues, and substrate scope have been described in detail. These mechanistically distinct approaches all allow complete conversion of Cys to Dha in proteins, though each method varies in scope.

The most general of these methods—the bis-alkylation–elimination with reagent **22**—was applied successfully to three model proteins, including both an enzyme and an antibody. Reagent **22** is stable, simple to prepare, and easy to handle. The elimination of cysteine to dehydroalanine using **22** is also straightforward. The selectivity of **22** can be compared to haloacetamides—a class of reagents long used for selective alkylation of cysteine.[40] To demonstrate the scope and utility of **22**, dehydroalanine was incorporated into the camel single-domain antibody cAb-Lys3, allowing subsequent conversion to GlcNAc cysteine. This is a rare example of the site-selective chemical glycosylation of an antibody.[90–96]

More generally, dehydroalanine itself is a target of synthesis (*e.g.* in the synthesis of lantibiotics) and also a precursor to many other post-translational modifications (Scheme 1). For many of these modifications there are currently no known methods for their synthesis using recombinant expression techniques. The syntheses of dehydroalanine described in this report should enable access to many of these modified proteins.

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