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One-pot, chemoselective desulfurative functionalization of cysteine containing peptides using pyridinium salts†

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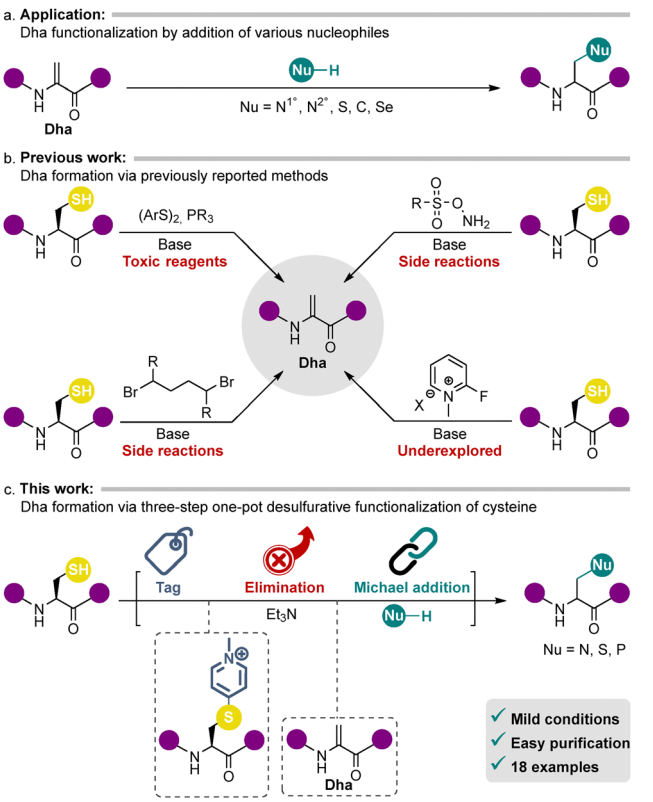
Peptide and protein modifications, especially late-stage derivatization, are invaluable for the synthesis of new pharmaceuticals. Currently, cysteine-mediated peptide modification is mainly limited to bioconjugation and disulfide formation. Therefore, exploration of new cysteine mediated peptide modifications is of great interest. Herein, we present a practical strategy for the three-step, one-pot desulfurative functionalization of cysteine containing peptides. The use of a pyridinium salt enables diverse and selective functionalization with an array of nucleophiles such as amino acid side chains, pharmaceuticals and macrocyclizations. This method allows for easy and diverse late-stage modification of peptides enabling the discovery and synthesis of new pharmaceuticals.

Peptides and proteins are vital in all living systems, where peptides are essential for physiological and biochemical processes and proteins are responsible for cellular structure and function.[1] New methods for modifying and functionalizing peptides have led to a marked increase in the number of peptide-based pharmaceuticals and drugs on the market over the last few decades.[2] Out of the 20 canonical amino acids, only those bearing reactive side chains such as lysine, cysteine, tyrosine or tryptophan are routinely used for chemical modifications.[3] The high nucleophilicity of the thiol group and its low natural abundance makes cysteine particularly valuable for functionalization,[4] typically *via* S-functionalizations such as alkylations/arylations and disulfide formations.[5]

In contrast, transformations involving deletion of the reactive thiol group are relatively unexplored.[4,6] Dehydroalanine (Dha) is a non-natural, yet biologically important, amino acid that has been widely used as a synthetic precursor for peptide/protein functionalizations *via* Michael additions at the electrophilic β-carbon (Scheme 1a).[7,8] Dha can be accessed by several methods[9] (Scheme 1b) including the reductive elimination of cysteine disulfides in the presence of electron-rich phosphines such as HMPT.[10] Oxidative elimination *via* the formation of sulfonium salts has also been reported,[11] although this approach



Scheme 1 Dha functionalization *via* Michael addition and strategies for Dha formation. (a) Conjugate addition of nucleophiles to Dha enables diverse functionalization. (b) Reported methods for Dha formation often rely on toxic reagents, suffer from side reactions, or remain underexplored. (c) This work involves a mild, one-pot, three-step desulfurative functionalization of cysteine with broad scope and simplified purification.

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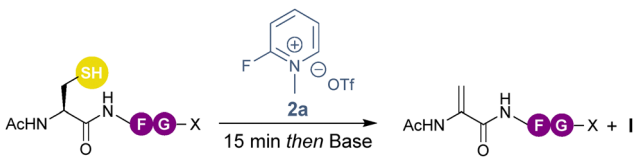
is susceptible to side reactions with amino acid residues such as histidine, lysine, methionine, aspartate and glutamate.[12] The thiol can also be bis-alkylated and eliminated using 1,4-dihalobutane derivatives[13] on small peptides or proteins with a single available cysteine. However, substrates containing more than one cysteine are burdened by side-reactions due to cross-linking, intramolecular cyclization or stapling.[14]

In 2011, Davis and co-workers disclosed a single example using Mukaiyama's reagent (2-chloro-1-methylpyridinium iodide) to promote elimination in moderate yield.[12] Since then, pyridinium salts have been elegantly employed to selectively "tag" cysteines and by tuning the pyridinium salts "detagging" with thiol nucleophiles can also be achieved.[15] Recognizing the potential of combining the high chemoselectivity of the cysteine-pyridinium tagging reaction, with the synthetic utility of Dha, we set out to develop a telescoped three-step one-pot desulfurative method for the straightforward, mild and selective late-stage functionalization of cysteine containing peptides (Scheme 1c). This approach enables the high-yielding modification of peptides with a variety of nucleophiles including pharmaceuticals and macrocyclizations.

Our initial experiments employed peptide **1a** (Ac-Cys-Phe-Gly-NH_2) and pyridinium **2a** as model substrates and DBU as the base (Table 1, entry 1). Although full consumption of **1a** was observed, a complex mixture of side-products was obtained. The main product was isolated and identified as thioacetate **I** (Fig. S1–S6, ESI†) likely formed through DBU-mediated *S*-arylation followed by deprotonation of the terminal acetamide and *N*-aryl transfer (Scheme S1, ESI†). To mitigate production of this side product, a variety of bases were tested at either 20 °C or 60 °C (Table S1, ESI†). Of these, only DABCO afforded high-selectivity towards **3a**, with complete conversion after 16 h.

By raising the temperature to 37 °C, the reaction time could be reduced to 7 h (Table 1, entries 2 and 3). Next, different solvents were screened (Table S2, ESI†) and we were pleased to note that the elimination rate to form compound **3a** was significantly enhanced in an aqueous buffer solution (Table 1, entry 4). Additionally, the solubility of the product was greatly improved. To assess the compatibility of the method with more realistic peptide substrates featuring a free C-terminus, we screened the carboxyl-containing compound **1b** (Table 1, entry 5). Notably, this had no influence on the formation of **3b**. Next, several bases were evaluated under aqueous conditions at 37 °C (Table 1, entries 6–8), all of which afforded full conversion (>99%). Any of these conditions could potentially be employed and NaOH was selected for further studies due to its low cost (Table 1, entry 7).

Table 1 Optimization of reaction conditions for generation of the Dha intermediates **3a** and **3b**[a]



1a, X = NH_2
1b, X = OH

3a, X = NH_2
3b, X = OH

Entry	Peptide	Solv.	Base	Temp., Time	Conv.[b]
1	**1a**	DMF	DBU	20 °C, <0.5 h	**3a** (0)
2	**1a**	DMF	DABCO	20 °C, 16 h	**3a** (>99)
3	**1a**	DMF	DABCO	37 °C, 7 h	**3a** (>99)
4	**1a**	H_2O[c]	DABCO	37 °C, 2 h	**3a** (>99)
5	**1b**	H_2O[c]	DABCO	37 °C, 2 h	**3b** (>99)
6	**1b**	H_2O[c]	DBU	37 °C, 1 h	**3b** (>99)
7	**1b**	H_2O[c]	NaOH[d]	37 °C, 1 h	**3b** (>99)
8	**1b**	H_2O[c]	Et_3N	37 °C, 2.5 h	**3b** (>99)

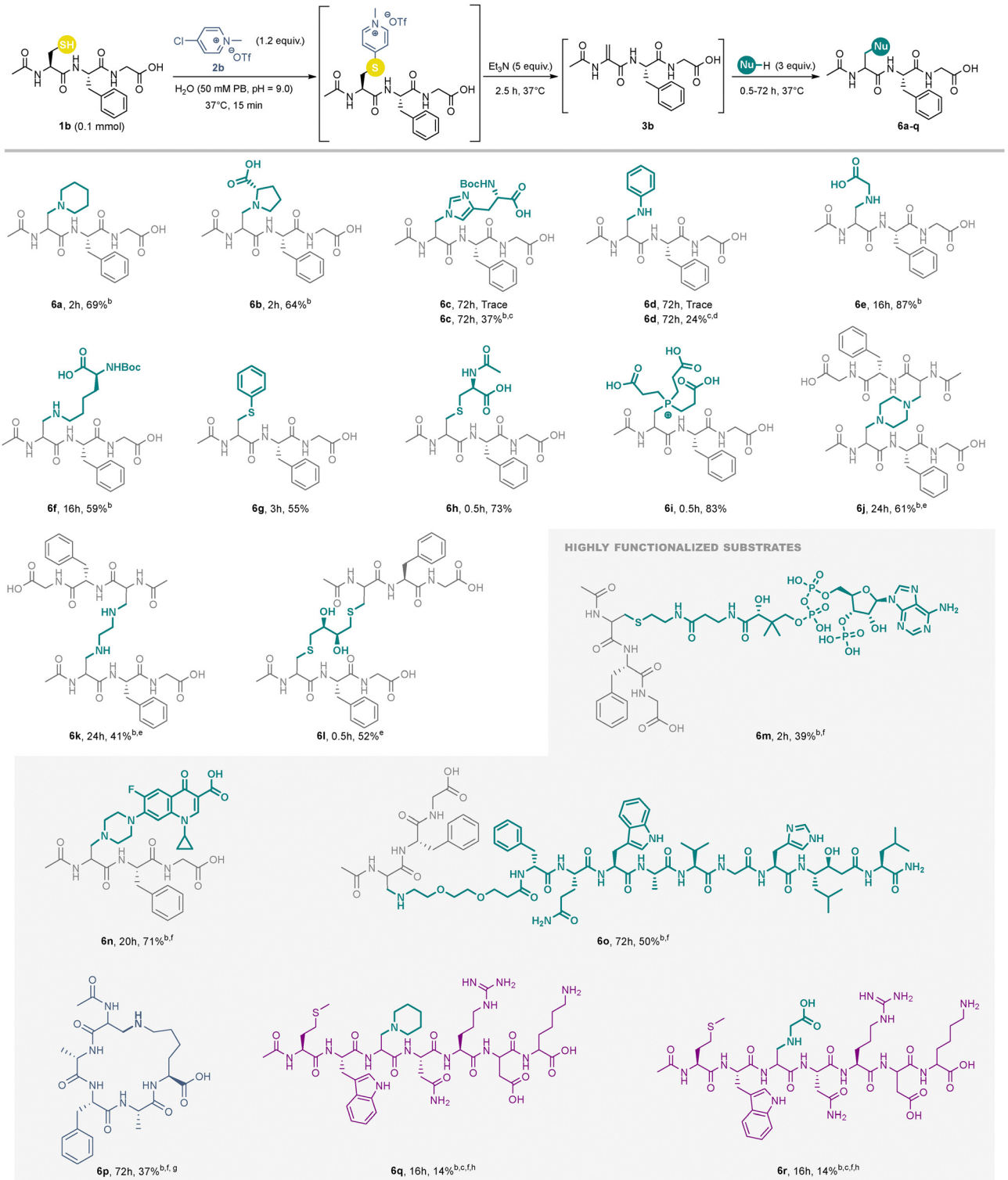
[a] Reaction conditions: **1** (0.025 mmol) and **2a** (0.03 mmol) for 15 min followed by base (0.03 mmol), solvent (0.2 mL). [b] Conversion to **3** based on the ratio of pyridinium tagged **1** and compound **3** determined by HPLC-UV analysis. [c] H_2O (50 mM PB, pH = 9.0). [d] 10 equiv. of base.

With the elimination step optimized, we turned our attention to the subsequent Michael-addition functionalization. Using piperidine as a model nucleophile, the tertiary amine containing peptide **6a** was successfully isolated in 40% yield (Scheme 2 and Table S3, ESI†). However, this reaction also generated a side-product, which was isolated and characterized as the thioether dimer **II** (Fig. S8–S10 and Scheme S2, ESI†). This is presumably formed by competing base-mediated elimination and hydrolysis of the pyridinium-tagged intermediate, leading to formation of **1b** and **3b**, which then undergo a thia-Michael addition to give **II**. Reducing the equivalents of base did not influence formation of **II**, but the use of the less electron-deficient pyridinium **2b** completely suppressed its formation (Table S3, ESI†) affording **6a** in 56% yield. Furthermore, the yield of **6a** could be increased to 69% (over three-steps) by changing the base to Et_3N (Scheme 2, Table S3 and Fig. S1–S4, ESI†).

With these optimized conditions in hand, we next investigated the nucleophile scope of for the desulfurative functionalization of **1b** (Scheme 2). We began by examining the secondary amine containing amino acid proline, which afforded 64% of the branched peptide **6b**, a yield comparable to the model reaction. Histidine was also found to be a viable substrate despite the lower nucleophilicity of the imidazole side chain,[16] yielding 37% of **6c**. The reaction could also be extended to aniline, affording a moderate yield of the *N*-aryl amine derivative **6d** (24%). Primary amines, including the glycine N-terminus and the lysine side chain reacted efficiently to furnish **6e** and **6f** in 59% and 87% yield, respectively, highlighting the potential for both N-terminal and side-chain peptide conjugation. However, amino acids bearing less nucleophilic side chains (tryptophan, serine, tyrosine, and arginine), did not yield the corresponding products (Fig. S13–S16, ESI†).

Beyond amines, thiols and phosphines were readily incorporated under the same conditions delivering conjugates **6g–6i** in good yields (55–83%). To explore peptide dimerization, primary and secondary diamines as well as a dithiol were investigated. Gratifyingly, all three nucleophiles produced dimeric peptides **6j–l** in 41–61% yields.

To further investigate the reaction compatibility with highly functionalized substrates, three complex nucleophiles were selected for interrogation (Scheme 2). First, coenzyme A[18] was used to probe reactivity with nucleotide-derived substrates, yielding **6m** in 39%. Next, ciprofloxacin[17] was employed to



Scheme 2 Scope of nucleophiles in the one-pot desulfurative functionalization of peptides using pyridinium salt **2b**[a]. [a] Isolated yield refers to a 1 : 1 mixture of diastereomers except for **6b** (3 : 1) and **6p** (7 : 1). [b] Isolated as TFA salt. [c] 10 equiv. of the nucleophile. [d] 74% purity based on UV (Fig. S27, ESI†). [e] 0.5 equiv. of the nucleophile. [f] Conditions in ESI.† [g] Peptide **1c** was used. [h] Peptide **1d** was used.

assess compatibility with drug-like structures, affording the novel antibiotic-peptide conjugate **6n** in 71% yield. Then, to examine the possibility of cross-linking larger peptide-based structures, PEG2-RM26 (a GRPR-targeting antagonist[19]) was functionalized to give **6o** in 50% yield. Employing peptide **1c**, a macrocyclization strategy was explored with lysine as a linchpin motif, affording the 19-membered macrocycle **6p** in a moderate 37% yield. To explore an additional challenging

substrate, peptide **1d** containing multiple competing nucleophilic groups and a sterically hindered cysteine residue was reacted with piperidine and glycine. In both cases, excellent conversion to the Dha derivative was observed, however the subsequent Michael addition was sluggish and the peptide derivatives **6q** and **6r** were both isolated in a yield of 14%. In the former case, separation of the **6q** from the intermediate dehydroalanine (Dha) species was problematic. In contrast, formation of **6r** was complicated by the formation of side-products **III** and **IV** (see ESI†), due to the use of an excess of **2b** (1.5 equivalents), highlighting the need for careful reagent control when using multifunctional substrates.

In conclusion, we have developed a mild, three-step one-pot desulfurative functionalization of cysteine *via* pyridinium activation. The method exhibits broad nucleophile scope, enabling efficient conjugation with primary and secondary amines, thiols, and phosphines (14–87% yields). A range of amino acid residues including lysine, proline, cysteine, and N-terminal amines are tolerated, and the reaction proceeds smoothly with complex, functionalized substrates. Notably, the strategy supports both intermolecular conjugation and macrocyclization. This platform offers a versatile approach for selective late-stage peptide modification and holds promise for applications in peptide–drug conjugates and chemical biology. Efforts to expand this chemistry to other amino acids and peptides are ongoing and will be reported in due course.

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Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

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