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Tunable heteroaromatic azoline thioethers (HATs) for cysteine profiling†

Kuei C. Tang,[a] Sean M. Maddox,[bc] Keriann M. Backus [bc] and Monika Raj *[a]

Here we report a new series of hydrolytically stable chemotype heteroaromatic azoline thioethers (HATs) to achieve highly selective, rapid, and efficient covalent labeling of cysteine under physiological conditions. Although the resulting cysteine–azoline conjugate is stable, we highlight traceless decoupling of the conjugate to afford unmodified starting components in response to reducing conditions. We demonstrated that HAT probes reverse the reactivity of nucleophilic cysteine to electrophilic dehydroalanine (Dha) under mild basic conditions. We demonstrated the umpolung capability of HAT probes for the modification of cysteine on peptides and proteins with various nucleophiles. We demonstrated that HAT probes increase the mass sensitivity of the modified peptides and proteins by 100 fold as compared to the classical methods. Finally, we extended the application of HAT probes for specific modification of cysteines in a complex cell lysate mixture.

Introduction

Chemical modification of proteins by small molecules in a selective manner is a powerful approach for drug design, imaging, chemoproteomic studies and activity based protein profiling (ABPP).[1–7] The key feature of the bioconjugation approach is the requirement of mild reaction conditions and selectivity for one particular amino acid in a plethora of all other reactive amino acids. Cysteine is the amino acid of choice for these modifications because of its low abundance (1.7%), high nucleophilicity of the side chain and important roles it plays in various biological processes including catalysis.[8,9] Various electrophiles have been reported for tagging cysteines such as haloacetamides (**IAA**),[10] epoxides,[11] sulfonate esters,[12] chloro- and acyloxymethyl ketones,[13–15] fluorobenzene,[16] aryl halides,[17] Michael acceptors[18,19] and heteroaromatic sulfones (Fig. 1a).[20–23] However, poor hydrolytic stability of probes or resulting conjugates, low reactivity and cross-reactivity with other amino acids[24–26] lead to the development of several metal-free and transition metal-mediated cysteine bioconjugation approaches by Davis, Bernardes, Pentelute, Wong, and others.[25–43]

Bioconjugation reactions with cleavable linkers have recently gained considerable attention due to their wide application in many research fields such as protein immobilization, drug development,[44,45] and proteomics.[46,47] Only a few compounds are available for cleavable cysteine-specific modification, including Ellman's reagent,[48,49] bromomaleimides,[50] bromopyridazinediones,[51] electron deficient acetylenes,[52] 5-methylene pyrrolones,[53] 4-substituted cyclopentenones[54] and recently discovered isoxazoliniums (Fig. 1b).[55] Despite all these advances, only ~17% of cysteine in the entire proteome has been identified so far.[56,57] Moreover, the resulting bioconjugates exhibit poor mass detection sensitivity limiting their applications in the identification of low abundant cysteine in the proteome. Therefore, there is a great need to develop new highly efficient cysteine selective modification methods with cleavable linkers using easily accessible reagents with high stability, distinct and tunable selectivity and reactivity that increases the mass detection sensitivity of the resulting conjugates that would aid in the identification of low abundant cysteines in the proteome.

In this study, we elucidate specific structural features of a new chemotype termed heteroaromatic azoline thioether (HAT) that is easily tunable, hydrolytically stable, and highly reactive and selective for Cys, and increases the mass detection sensitivity of resulting conjugates (Fig. 1c). We elucidate that HAT, a largely unexplored cysteine-reactive electrophilic group undergoes chemically triggered decoupling to generate the native unchanged coupling partners in a "traceless" manner under mild conditions (Fig. 1c). Another unique feature of HAT probes is their ability to reverse the reactivity of nucleophilic Cys into an electrophilic moiety (Fig. 1c). This inversion of reactivity enables selective modification of proteins with a variety of nucleophiles. These innovative HAT tools for probing cysteine would augment existing detection methods

[a]*Department of Chemistry, Emory University, Atlanta, GA 30322, USA. E-mail: monika.raj@emory.edu*

[b]*Department of Biological Chemistry, David Geffen School of Medicine, UCLA, Los Angeles, CA, 90095, USA*

[c]*Department of Chemistry and Biochemistry, College of Arts and Sciences, UCLA, Los Angeles, CA, 90095, USA*

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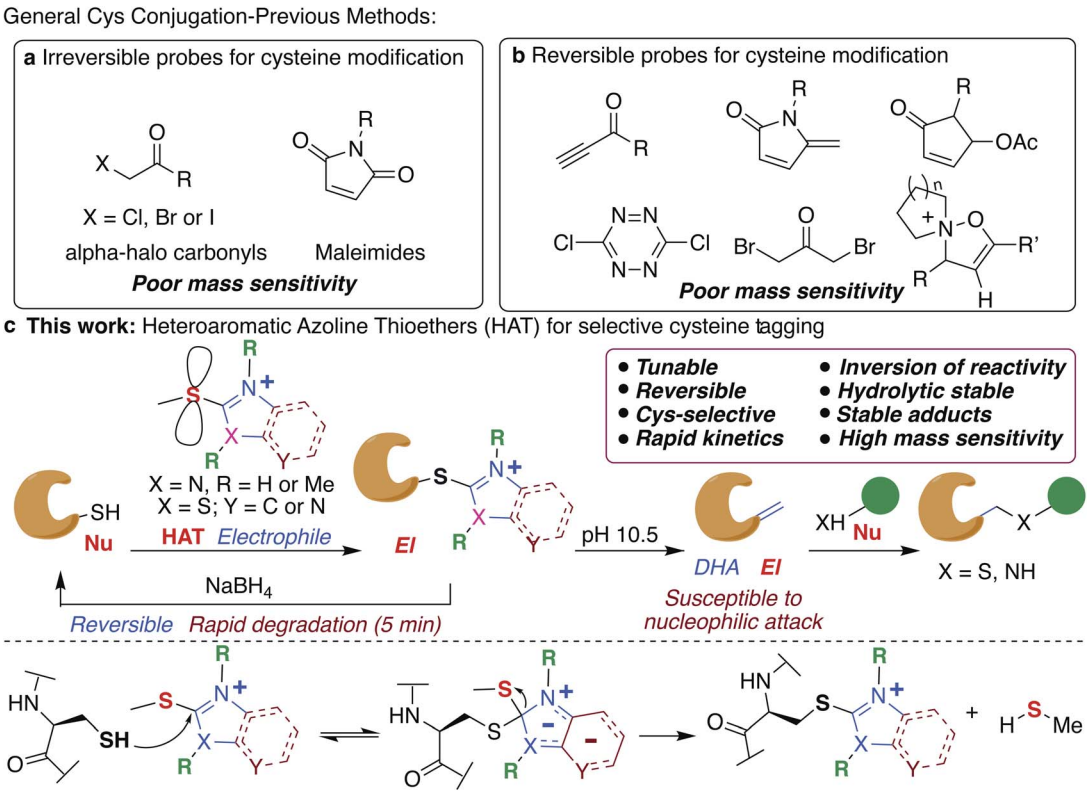

Fig. 1 Reagents for selective tagging of cysteine. (a) Classical reagents for cysteine modification. (b) Reagents for cleavable cysteine modification. (c) This work: a general strategy for cysteine modification using HAT probes, cleavable under reduced conditions, umpolung capability under basic conditions and increasing the mass sensitivity of the labeled fragments. Possible mechanism of cysteine modification with HAT probes by S_NAr substitution to generate stable adducts.

and will dramatically expand the toolbox for bioconjugation, proteome profiling, for targeting otherwise undruggable protein targets and identifying protein partners.

Results and discussion

Design of HAT probes

Thioethers are privileged scaffolds found in various FDA approved drugs and are the third most exemplified constituent (8.8%) of sulfur containing drugs including representative pharmaceuticals with heteroaromatic units such as cimetidine, imuran, ranitidine, azathioprine, butoconazole and many others.[58] Some of the heteroaromatic thioethers such as 2-(thioalkyl)benzoxazole act as prodrugs and undergo S-oxidation during metabolism forming active sulfone drugs.[59]

Although thioethers are hydrolytically stable as compared to their oxidized sulfinyl and sulfone analogs, they have never been explored for protein modification due to their intrinsic low reactivity. Here we display systematic efforts to tune the reactivity and selectivity of thioethers thus expanding their utility for the selective modification of cysteine in proteins as a reversible cleavable linker and chemoproteomic profiling.

We tune the thioether probes to find a sweet spot between high reactivity, and high hydrolytic stability and selectivity for Cys. We achieved this by (i) exploring different heteroatoms on azoline-thioethers, (ii) changing the heterocyclic ring to azole, (iii) by fusing aromatic moieties to azoline-thioethers to generate heteroaromatic azoline-thioether (HAT) probes, (iv) varying the S-oxidation state, and (v) by evaluating different methylation states of azoline-thioethers and heteroaromatic azoline-thioether (HAT) probes (Fig. 2a and b). Despite various developments in the field of Cys modification, iodoacetamide (**IAA**) is still commonly used for the modification and profiling of Cys.[60] This is mainly due to its high hydrolytic stability and easy synthesis of **IAA** derivatives although it exhibits slow reactivity and cross-reactivity with lysine. Throughout the manuscript, we will compare our optimized HAT probes with well-known **IAA** and oxidized sulfone analogs in terms of selectivity, reactivity, stability, ionization potential, and reversibility.

Heteroatom core optimization

We started our initial investigation by reacting a peptide FKVCF (4 mM, 1 equiv.) containing multiple nucleophilic residues such as N-terminus, Lys and Cys with azoline-thioether probes **1** (40 mM, 10 equiv.) with various heteroatoms in the azoline ring (Synthesis of probes, ESI Fig. 1†). The reaction was conducted in phosphate buffer (NaP, pH 7.5, 10 mM) at room temperature under air for 3 h. Remarkably, the reaction with 2-methylthio thiazoline **1a** labeled only the Cys residue without any modification of the N-terminus and lysine side chain due to the low

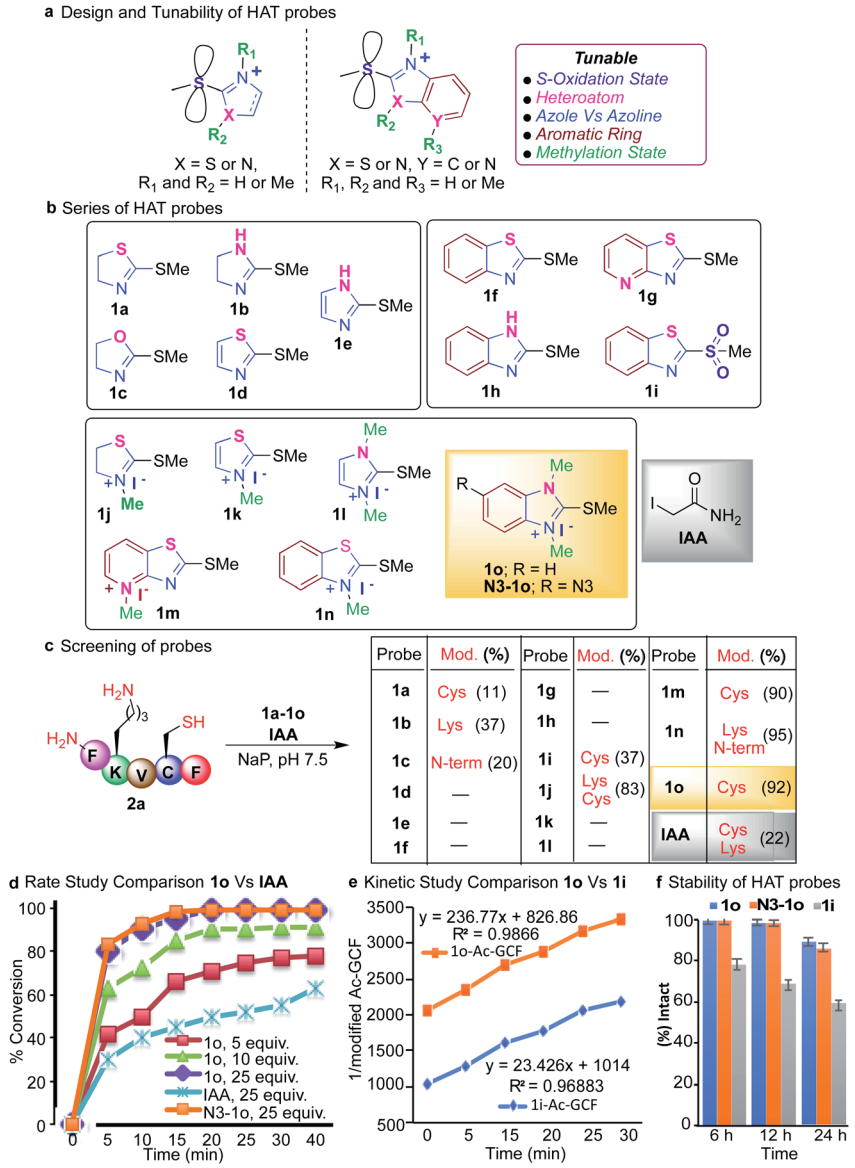

Fig. 2 Tunable and stable HAT probes for targeting cysteine. (a) Design of hydrolytically stable thioether probes to tune their reactivity and selectivity for modification of cysteine. (b) Structures of a variety of HAT probes with different modifications to investigate their reactivity and selectivity for cysteine. (c) Screening of probes (40 mM, 10 equiv.) by using peptide FKVCF (4 mM, 1 equiv.) with all the nucleophilic residues and comparison with well known **IAA** and sulfone probe (**1i**) (40 mM) under physiological conditions (NaP (10 mM), pH 7.5, 25 °C) for 3 h. Probe **1o** showed high selectivity and reactivity for cysteine as compared to sulfone probe **1i** and **IAA**. (d) Observed rate of the modification of cysteine of the peptide Ac-GCF **2b** (0.003 mM) with **1o** (5–25 equiv.), its azide analog **N3-1o** (0.6 mM, 25 equiv.) and **IAA** (0.6 mM, 25 equiv.) in 10 mM phosphate buffer (pH 7.5, 25 °C) at different time intervals. (e) Kinetics study comparison of probe **1o** (0.973 mM) and **1i** for labelling peptide Ac-GCF **2b** (0.973 mM) showed a 10 fold faster rate of probe **1o** for cysteine modification as compared to sulfone analog **1i** (0.973 mM) under physiological conditions (NaP (10 mM), pH 7.5, 25 °C). In both (d) and (e), each time point represents an average of three independent experiments. (f) High stability of probe **1o** (38.75 mM) and its azide analog **N3-1o** (38.75 mM) as compared to sulfone probe **1i** (38.75 mM) under physiological conditions (NaP (10 mM), pH 7.5, 25 °C).

electrophilicity of **1a**.[61] 2-methylthio imidazoline **1b** labeled Lys, due to the ability of the imidazole of **1b** to act as a base and deprotonate the lysine side chain. 2-Methylthio oxazoline **1c** labeled the N-terminus of peptide FKVCF because the pK_a of the side chain of lysine ~10.6 is higher than the pK_a of the N-terminus ~9.6 and therefore, the side chain of lysine remained protonated under the physiological conditions and labeling of lysine was not observed as determined by MS/MS (Fig. 2c and ESI Fig. 2†).[61] The study showed that heteroatoms play a very important role in tuning the reactivity of azoline-thioether probes towards various nucleophiles. Replacing the sulfur atom in azoline with an oxygen or nitrogen atom completely switches the chemoselectivity as reported in our previous study.[61] Conversely, substituting the heterocyclic ring azole for azoline, 2-methylthio thiazole **1d** and 2-methylthio imidazole **1e** eliminated the reactivity for Cys (Fig. 2c). These studies clearly

showed that modulation of the heterocycle immensely influenced the selectivity and reactivity of thioethers towards various nucleophiles.

To assess the potential to tune the reactivity towards cysteine, we evaluated the reactivity and chemoselectivity of 2-methylthio benzothiazoline **1f**, 2-methylthio pyridthioazoline **1g** and 2-methylthio benzoimidazoline **1h** where a benzene or pyridine ring is directly fused to the heterocycle thiazoline and imidazoline of thioethers (Fig. 2b, Synthesis of probes, ESI Fig. 1†). The fusion of aromatic rings drastically reduces the solubility of the azoline-thioether probes in aqueous buffer conditions and thus no reactivity was observed with peptide FKVCF in phosphate buffer at pH 7.5 (Fig. 2c). We next examined the influence of different oxidation states of sulfur on 2-methylthio benzothiazoline and the resulting sulfone analog **1i** enhanced aqueous solubility and imparted Cys reactivity as confirmed by the reaction with peptide FKVCF (4 mM) followed by MS/MS analysis (37% conversion, **1i** probe (40 mM, 10 equiv.) in 3 h, Fig. 2c, ESI Fig. 2†).[62] However, reactive sulfones are also susceptible to degradation in aqueous solution.[59]

Methylation of heteroatom-core optimization

One common way to tune the reactivity of electrophilic probes is by substituting aromatic rings with electron withdrawing or electron releasing groups. The reactivity of electrophilic probes varies tremendously by the attachment of different electron withdrawing substituents on the aryl ring, with the nitro group showing very high reactivity and the fluorine group with modest reactivity as shown in a previous study.[20] Therefore, probe derivatives such as affinity tags (alkynes or azides, biotin) or dye-derivatives that are obtained by the substitution at the aromatic ring showed a huge disparity in their reactivities as compared to the model probes used for the optimization studies. In fact, affinity tag-derived probes showed significantly reduced reactivity as compared to the highly reactive nitro analogs utilized for model studies.[20] We hypothesized that by tuning the reactivity of the core structure of the probe, one can obtain probes with predictable reactivity and selectivity independent of the aryl or alkyl substitutions. Therefore, we carried out methylations of the heteroatoms of the core structure of heteroaromatic azoline-thioether (HAT) probes to tune their reactivity and selectivity (Fig. 2b, Synthesis of probes, ESI Fig. 1†). The methylation of HAT probes also increases their solubility in an aqueous solution. To obtain the right balance between the high reactivity of the HAT probes, their hydrolytic stability, and selectivity, a small series of thioether probes were synthesized with various methylation states (**1j–1o**) (Fig. 2b). Next, we screened the probes **1j–1o** (10 equiv., 40 mM) with peptide FKVCF (4 mM) under physiological conditions (NaP, pH 7.5, 10 mM). Probe **1j** obtained from the *N*-methylation of **1a** modified both Cys and Lys due to the increased electrophilicity as confirmed by MS/MS (Fig. 2c and ESI Fig. 3†). Probe **1n** showed modification of Lys and N-terminus because the conjugate obtained by reaction with cysteine is reversible and either reacts with lysine and N-terminus to generate a stable product or with the released methanethiol to generate the unreacted starting material[63] (Fig. 2c and ESI Fig. 3†).

Interestingly both **1m** and **1o** showed high reactivity and selectivity for Cys (90–92% conversion, Fig. 2c and ESI Fig. 4†). Importantly, a comparison experiment performed with **IAA** under identical reaction conditions, showed modification of both Lys and Cys residues of peptide FKVCF (22% conversion) demonstrating the poor selectivity of **IAA** towards Cys (Fig. 2c and ESI Fig. 5†). These studies showed that methylations of heteroatoms increase aqueous solubility and induce huge variations in the HAT reactivity, and thus are capable of inducing selectivity for a particular target by rational tuning and provide a simple strategy to tune the core structure across the aromatic heterocyclic scaffold. Probe **1o** has previously been reported for the synthesis of carbodicarbenes but has never been explored for selective peptide and protein modification.[64] The optimization studies revealed that the reaction between peptide Ac-GCF (3.75 mM) and HAT probe **1o** (25 equiv., 93.75 mM) selectively labels Cys and proceeds most efficiently in phosphate buffer (NaP, pH 7.5, 10 mM) at room temperature, resulting in the formation of a stable coupling product with >99% conversion in 3 h (ESI Fig. 6†).

Under optimized conditions, **IAA** generated 70% conjugated product and sulfone analog **1i** generated 53% conjugated product (ESI Fig. 6†). To characterize the **1o-Cys** coupling product, reaction with a model compound 2-(Boc-amino)ethanethiol was carried out on a large scale under the optimized conditions. The resulting product was isolated and cysteine labeling by **1o** was confirmed by NMR (^{1}H and ^{13}C) (ESI Fig. 7†). In contrast, no product was observed with peptide GAF-OMe (w/o Cys), thus reconfirming the high chemoselectivity of HAT probe **1o** for Cys (ESI Fig. 8†).

Rate study of labeling cysteine with the HAT probe

Next, we monitored the reaction between peptide Ac-GCF **2b** (3 mM) and HAT probe **1o** (5–25 equiv.) after regular intervals of time and compared it to the corresponding reaction with **IAA** (25 equiv. 0.6 mM) under optimized conditions (NaP, pH 7.5, 10 mM) (Fig. 2d and ESI Fig. 9†). The formation of the coupling product was analyzed using HPLC and MS. The reaction proceeded with fast kinetics and more than 80% conversion to the cysteine modification product was observed in 5 min using 25 equiv. of the **1o** probe (Fig. 2d and ESI Fig. 9†). The comparison experiment with **IAA** (25 equiv.) showed a decreased product formation (30%) in 5 min. Finally, we carried out the rate studies with the azide-derivative of **1o**, (**N3-1o**) to determine the role of the substituent in modulating the rate of the reaction. The reaction with **N3-1o** (25 equiv.) showed a comparable reaction rate to **1o** (Fig. 2d and ESI Fig. 9†). The similar reactivity profile of **1o** and **N3-1o** reaffirms our initial hypothesis regarding the modulation of the core structure to obtain more predictable reactivity with various probe-derivatives. Next, the bioorthogonal reaction of **N3-1o** modified peptide, N3-1o-AcGCF **2b** with sulfo-DBCO-Biotin using strain promoted alkyne–azide cycloaddition (SPAAC) under physiological conditions (Nap, pH 7.5, 10 mM) generated the conjugated

product with full conversion as analyzed by ESI-MS (ESI Fig. 9†). This study showed the compatibility of the HAT–Cys coupling product with SPAAC thus applicable for proteome profiling and enrichment.

Next, we compared kinetics of the reaction of HAT probe **1o** (0.973 mM) and its oxidized sulfone analog **1i** (0.973 mM) with peptide AcGCF **2b** (0.973 mM) in buffer (NaP, pH 7.5, 10 mM) at room temperature. The results showed that **1o** ($k = 236.77\ M^{-1}\ S^{-1}$) is 10 times more reactive than **1i** ($k = 23.43\ M^{-1}\ S^{-1}$) (Fig. 2e and ESI Fig. 10†).

Stability of HAT probes

It is usually considered that highly reactive probes exhibit poor hydrolytic stability. Therefore, we sought to determine the stability of the HAT probes **1o** and **N3-1o** and compared them with sulfone analog **1i**. These probes (38.75 mM) were incubated in aqueous phosphate buffer (pH 7.5) under ambient conditions and monitored after regular intervals of time by HPLC. Surprisingly, more reactive probes **1o** and **N3-1o** are highly stable for 6 h without any observed degradation and the less reactive **1i** probe showed 24% degradation in 6 h. Only 14–17% hydrolysis of probes **1o** and **N3-1o** was observed in 24 h as compared to **1i** that showed 40% hydrolysis in 24 h (Fig. 2f and ESI Fig. 11†). These studies showed that HAT probes **1o** and **N3-1o** exhibit high reactivity and high selectivity towards cysteine and are hydrolytically more stable as compared to **1i**.[65,66] The HAT probe **1o** is bench stable for 3 months as a white solid, demonstrating robust properties for long-term storage.

Protein modification with HAT

Previous studies with carbon electrophiles such as chloroacetamide and sulfonate esters showed that the solution reactivity of electrophiles with peptides is often not predictive of reactivity observed with proteins due to the unique protein environment, which modulates the pK_a and reactivity of amino-acid side chains. To evaluate the selectivity and reactivity of HAT probes towards Cys with proteins, we carried out the reaction of all the HAT probes (**1a–1o**) with myoglobin (Mb) (w/o Cys). The modification of Mb was observed with all the reactive probes (**1a–1c**, **1j**, **1n**, and **IAA**) except **1i**, **1m** and **1o** thus clearly showing their high selectivity for Cys since Mb does not have the Cys residue thus corroborating with peptide screening data (Fig. 3a and ESI Fig. 12†). Most of the other HAT probes reacted with other nucleophilic amino acids of Mb such as Lys (ESI Fig. 13†). Probes **1d–1h** and **1k–1l** do not show any reactivity with Mb due to their low reactivity and less water solubility similar to peptide screening data (Fig. 3a). To determine the reactivity and selectivity of **1o** and **1m** for proteins, we carried out the reaction with both native insulin (w/o free Cys) and reduced insulin containing six free cysteine residues (two in chain A and four in chain B, ESI Fig. 14†). We observed full modification of all the cysteine residues in both chains A and B of reduced insulin by **1o** (>99% conversion) as compared to **1m** (chain A – 55% and chain B – 40%) under identical conditions (50 equiv. **1m**, 12 h, pH 7.5) as analyzed by MS (ESI Fig. 14†). The modification of native insulin was not observed with both **1o** and **1m** thus confirming high selectivity of **1o** and **1m** for Cys (ESI Fig. 14†).

Next, we studied and compared the selectivity of **IAA** for Cys using myoglobin (Mb). Under physiological conditions (NaP, pH 7.5, 10 mM), the reaction of Mb with **IAA** (100 equiv.) generated the Mb-conjugate with multiple modifications (Fig. 3a and ESI Fig. 15†). This showed that **IAA** is not highly selective for Cys and leads to the modification of other reactive nucleophiles on Mb including Lys, and N-terminus, thus reconfirming literature reports.[67–69] These studies showed that HAT probe **1o** is more reactive and highly selective for cysteine conjugation as compared to widely used **IAA** and all other HAT probes.

After a comprehensive study on the efficiency, chemoselectivity, scope, and stability of HAT probe **1o**-mediated cysteine modification, we further explored its applicability for other protein bioconjugation. Bovine serum albumin (BSA) with a single free cysteine residue was utilized for bioconjugation. Treatment of BSA (0.15 mM) with HAT probe **1o** (300 equiv.) in Nap 7.5 buffer at 25 °C for 8 h afforded modified protein **BSA-1o** in >99% conversion by LC-MS analysis (Fig. 3b and ESI Fig. 16†). For oxidized-cysteine-containing proteins, lysozyme, under the same reaction conditions, no modification was found. Reduction of lysozyme generated 8 free cysteines and reaction with **1o** modified 1–3 cysteine residues in reduced lysozyme (Nap 7.5 buffer at 25 °C for 8 h, >99% conversion) (Fig. 3b and ESI Fig. 16†). The high reactivity of the probe towards particular cysteines in lysozyme is due to easy surface accessibility and the microenvironment influencing the pK_a of cysteines making it more reactive. These results indicated that the HAT probe-mediated modification could be conducted with high efficiency and chemoselectivity on proteins.

Synthesis of HAT affinity tags for Cys bioconjugation

The effective bioconjugation reaction for enrichment should have the ability to attach affinity tags, thus we generated the azide functionalized HAT probe **N3-1o** (Synthesis of probes, ESI Fig. 1†). We then evaluated the reactivity of the azide-functionalized HAT probe **N3-1o** towards Mb, native insulin and reduced insulin (Fig. 3c and ESI Fig. 17†). Similar to the model probe studies with **1o**, azide-HAT probe **N3-1o** did not modify Mb and native insulin but modified all the cysteine residues on both chains A and B of reduced insulin under optimized conditions thus showing high selectivity for Cys as confirmed by LCMS and HPLC (>99%, Fig. 3c and ESI Fig. 17†). The strain promoted alkyne–azide cycloaddition (SPAAC) with sulfo-DBCO-Biotin further functionalizes **N3-1o**-modified insulin chains. The reaction products were analyzed by MS and the data showed dual labeling of all the free Cys residues on reduced insulin with full conversion (>99%, Fig. 3c and ESI Fig. 17†). This study also showed the compatibility of the HAT–Cys coupling product with SPAAC thus applicable for proteome profiling and enrichment.[70]

Selective tagging of Cys in a complex mixture

As a further demonstration of the high selectivity of HAT probe **1o** for Cys, we attempted tagging multiple proteolytic fragments in the same solution to test the potential of our method for

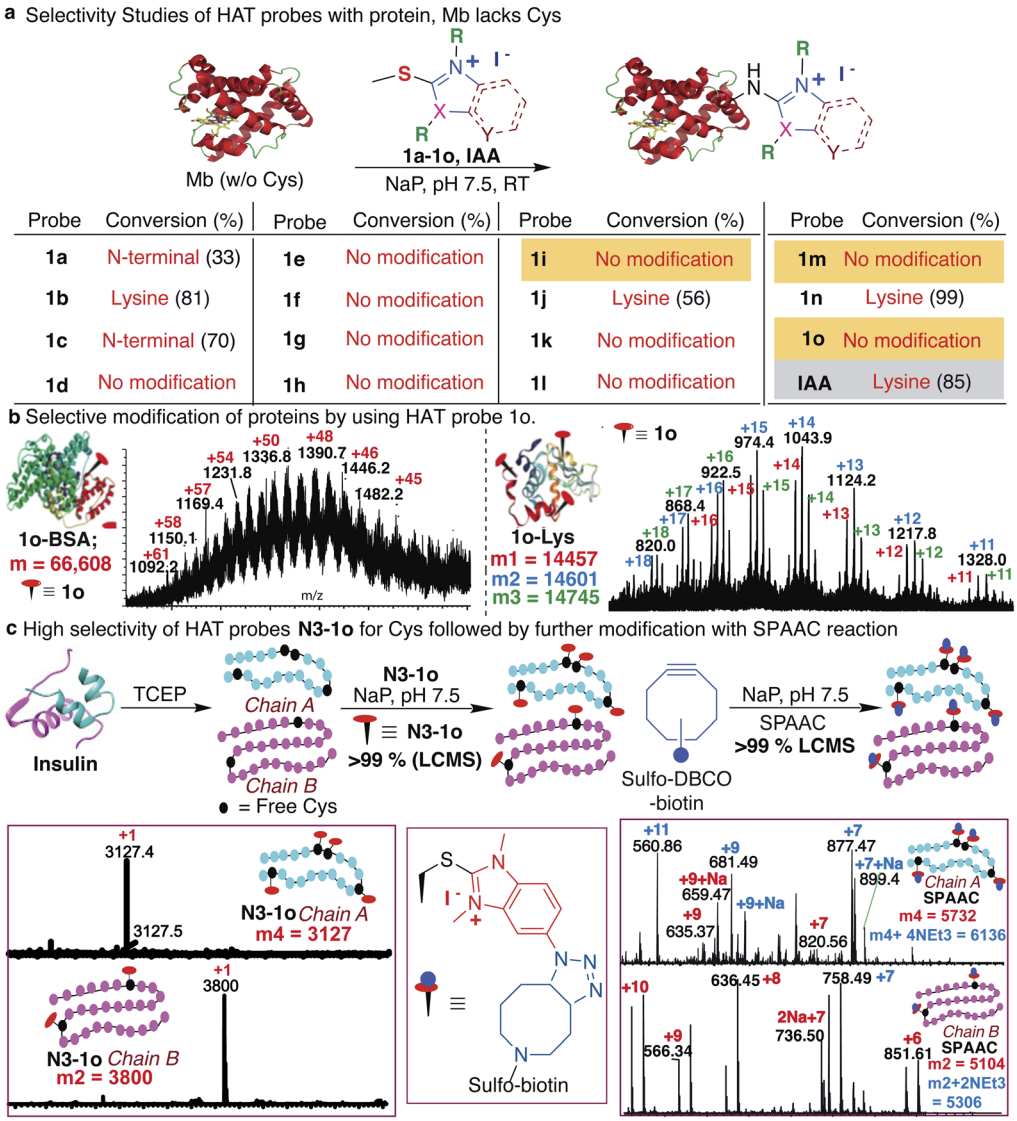

Fig. 3 HAT probes for protein functionalization. (a) Conjugation of myoglobin (Mb) with various HAT probes. Mb does not have any cysteine residue and probe **1o** showed no reactivity with Mb thus confirming high selectivity for cysteine. However, widely used cysteine selective probe **IAA** modifies the Mb. (b) Selective modification of cysteine in a protein BSA with one free cysteine and reduced lysozyme (1–3 cysteine modifications observed). (c) Modification of native and reduced insulin (0.15 mM) with HAT probe **N3-1o** (7.5 mM). Modification of all six free cysteines in reduced insulin and no reaction with native insulin (w/o free cysteine, disulfide form), confirms high selectivity of **N3-1o** towards cysteine. The strain promoted alkyne–azide cycloaddition (SPAAC) with sulfo-DBCO-Biotin further functionalized **N3-1o**-modified insulin chains. Reaction conditions: **N3-1o**-modified insulin protein (0.15 mM), sulfo-DBCO-Biotin (1.5 mM) under physiological conditions (NaP pH 7.5, 25 °C) for 4 h.

enrichment in a complex mixture. The mixture of proteolytic fragments obtained by CNBr cleavage of Mb, Cytochrome C and reduced insulin was incubated with HAT probe **1o** for 12 h under the optimized reaction conditions. The reaction was analyzed by LCMS and the data showed the tagging of only free Cys containing proteolytic fragments with **1o** in the reaction mixture (ESI Fig. 18†).

Reversibility and stability of the HAT–cysteine conjugation

Next, we determined the stability of the **1o**-conjugated peptide 1o-Ac-GCF **3b** under different reaction conditions by monitoring with HPLC. We demonstrated that peptide conjugate 1o-Ac-GCF **3b** is stable at low pH 3.5 for 48 h at both room temperature 25 °C and 40 °C and only 10% degradation was observed in 24 h at pH 7.5 (ESI Fig. 19†). Peptide conjugate 1o-Ac-GCF **3b** is also stable to a strong protein disulfide reducing agent tris(2-carboxyethyl)phosphine (TCEP) for 48 h without any decomposition (ESI Fig. 19†). Even though the HAT–peptide conjugate is stable under physiologically relevant conditions, we aimed to explore if the conjugate can be decoupled in a traceless manner to avoid any limitations associated with the irreversible inhibition of proteins. One way to achieve this goal is to invert the reactivity of the nucleophilic Cys residue into

electrophilic DHA, thus allowing reversibility by the attack of a nucleophile on the unchanged starting material. Indeed, exposure of the peptide conjugate 1o-Ac-GCF **3b** to reduction conditions in the presence of sodium borohydride (10 equiv., 25 °C, in NaP pH 7.5) led to its rapid degradation to unchanged peptide Ac-GCF **2b** (>99% conversion) in 5 min, as observed by HPLC and MS analysis (ESI Fig. 20†). Next, we applied the reversibility approach for insulin modified with **1o**; we subjected **1o**-modified insulin chains A and B to reducing conditions and within 5 min observed the complete reversibility to unmodified insulin chains A and B with full conversion (>99% Fig. 4a and ESI Fig. 20†). It is noteworthy that the reduction generated original protein in an unperturbed manner. The high selectivity and reactivity of the HAT probes for Cys under physiological conditions, and the ability to undergo rapid reversal in a traceless manner to regenerate unmodified protein, underscore the unique advantages of the HAT probes in bioconjugation and proteome profiling, thus also minimizing the limitations associated with producing irreversibly modified proteins.

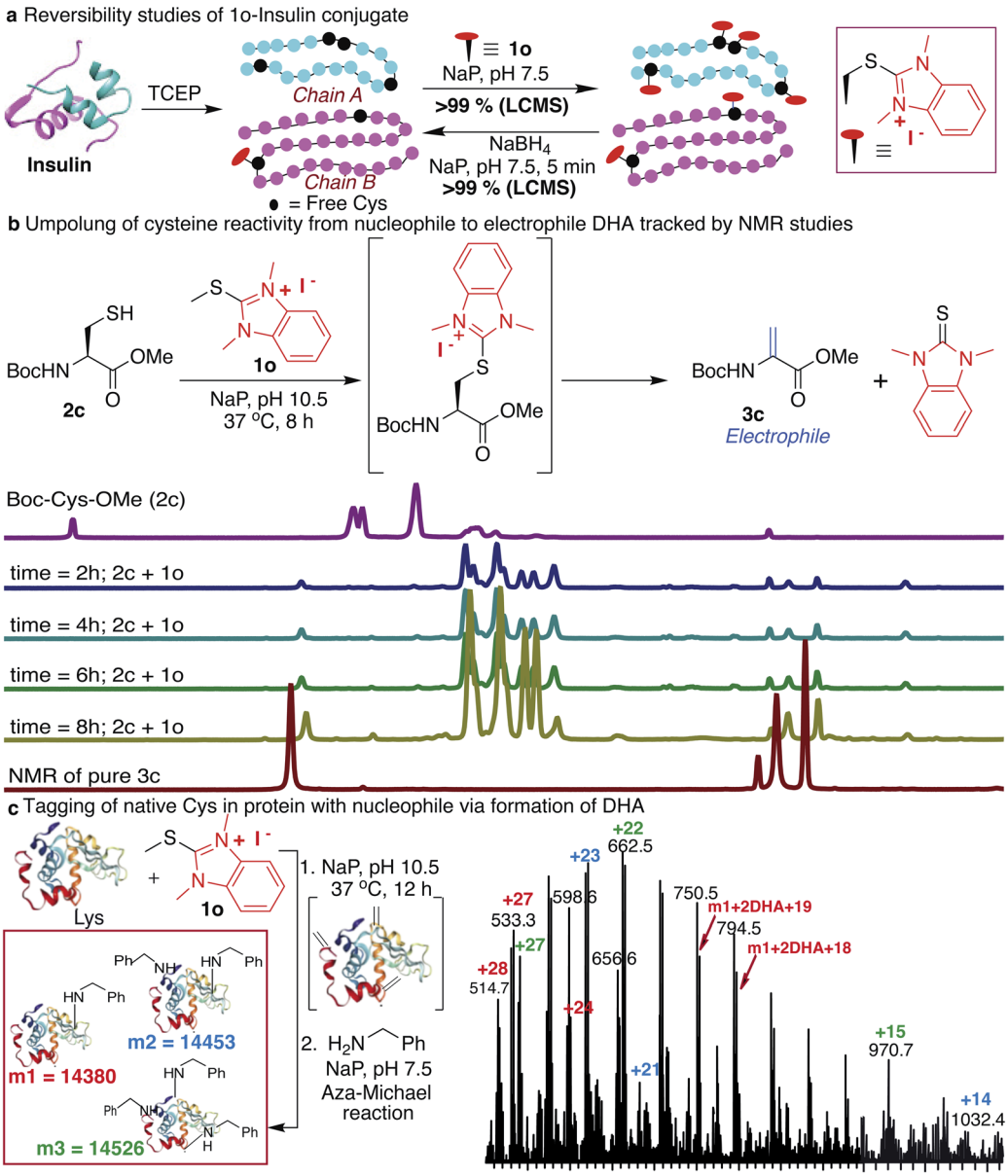

Fig. 4 Reversibility and inversion of the reactivity of cysteine by HAT probe **1o**. (a) Reversibility of the **1o**-protein conjugate (0.15 mM) in the presence of sodium borohydride (1.5 mM) in NaP (pH 7.5) in 5 min. (b) Conversion of peptide Boc-Cys-OMe **2c** directly to dehydroalanine DHA **3c** under basic conditions (NaP, pH 10.5, 8 h) with >99% conversion. ^{1}H NMR shows the formation of dehydroalanine (DHA) **3c** from the HAT–Cys conjugate in a one step process. (c) Conversion of free cysteine of protein lysozyme (0.15 mM) to DHA (conversion >99%) by incubating with probe **1o** (45 mM) at pH 10.5 for 12 h followed by the addition of amine (7.5 mM) by the aza-Michael reaction on DHA at pH 8.5 for 12 h to generate amine labeled protein (conversion 78%).

Reactivity inversion by HAT probes: reaction with nucleophiles

Many protein conjugation methods utilize the inherent nucleophilicity of Cys and carry out reactions with electrophiles. A different approach for modification at cysteine is obtained by inverting the reactivity of nucleophilic cysteine residue into electrophilic DHA, thus allowing for modification by various nucleophiles. This approach could also be applicable to capture the protein interaction partners by the formation of the covalent bond between the DHA on a peptide or protein and lysine of the interacting protein partner. We sought to achieve this goal by exposing Boc-Cys-OMe **2c** to basic conditions (NaP, pH 10.5, 37 °C) and observed the formation of dehydroalanine Dha **3c** directly *via* the **1o-Cys** modified intermediate as observed by NMR and LCMS (86%, 8 h, Fig. 4b, and ESI Fig. 21†). The spontaneous elimination of the **1o-Cys** conjugate under basic conditions generates a type 2 alkene, dehydroalanine Dha, which in principle serves as a handle for further conjugation with various cargoes such as polyethylene glycol (PEG) polymers, fluorophores, or affinity reagents by reaction with nucleophilic amines and thiols.[71–77] By using HAT probes we converted nucleophilic Cys of a peptide Ac-GCF **2b** to Ac-G(Dha)F **3d** (88% conversion)

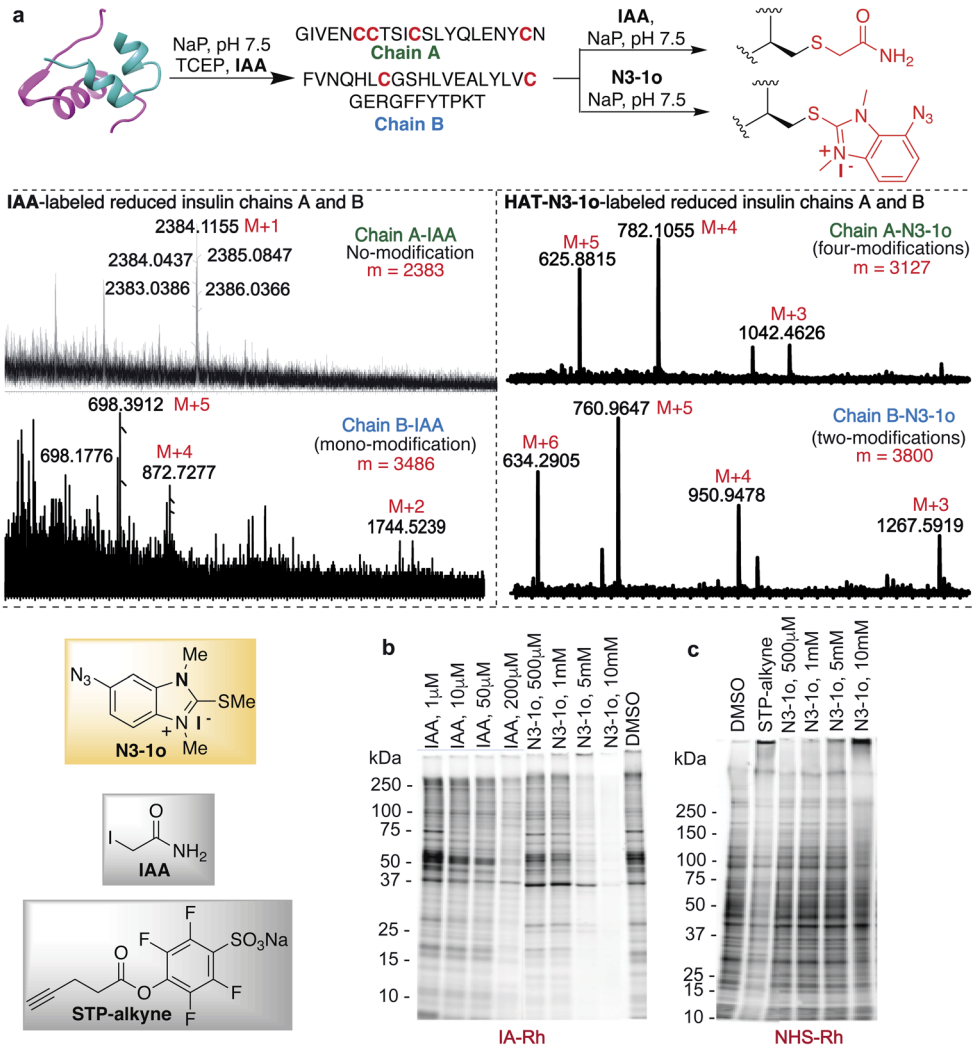

Fig. 5 HAT probe **1o** as mass sensitivity booster. (a) HAT probe **1o** significantly increases the mass detection sensitivity of a protein as compared to **IAA** tagged protein. Tagging with HAT probe **N3-1o** improves the detection of chain A of reduced insulin significantly (right MS trace). Chain A is not modified after TCEP reduction and labeling with **IAA** (left MS trace). Both chains A and B of reduced insulin are visible after tagging with the HAT probe, **N3-1o** (right MS trace). **1o** modified all 4 cysteines and 2 cysteines of chains A and B respectively. **IAA** modified only one cysteine of chain B under identical conditions. Reaction conditions: reduced insulin (0.15 mM), probes **1o** or **IAA** (50 equiv.) in NaP buffer at pH 7.5, room temperature for 8 h. (b) Chemoproteomic studies of the HAT **N3-1o** probe by gel-based competitive activity-based protein profiling (ABPP). In-gel fluorescence analysis of the HAT **N3-1o** probe at different concentrations (500 μM to 10 mM) and comparison with the cysteine reactive **IAA** probe (1–200 μM) followed by incubation and detection with IA-Rh in HEK293T cell lysate. Fading of bands with an increase in the **N3-1o** concentration showed labeling with cysteine. (c) In-gel fluorescence analysis of the HAT **N3-1o** probe at different concentrations (500 μM to 10 mM) and comparison with the lysine reactive **STP-alkyne** probe (1 mM) followed by incubation and detection with NHS-Rh. No fading of bands with increasing concentration of **N3-1o** indicates no reactivity with lysine.

followed by reactions with a variety of nucleophiles such as mercaptoethanol and benzylamine to generate thiol addition product **3e** and conjugated amine **3f** with >99% conversion (ESI Fig. 22†). Next, we converted the nucleophilic Cys on reduced protein lysozyme to DHA by treatment with probe **1o** at pH 10.5. We observed modification of three cysteines of the reduced lysozyme to DHA with >99% conversion. Next, we carried out labeling of DHA modified lysozyme with benzylamine by the aza-Michael reaction to generate amine-modified lysozyme with 78% conversion as analyzed by LCMS (Fig. 4c and ESI Fig. 23†).

HAT as mass sensitivity booster

One of the limitations with current methods of selective labeling of Cys is the difficulty in the characterization of resulting bioconjugates by MS due to the poor ionization of the labeled fragments.[76] This limitation is more prominent in complex mixtures leading to a major roadblock to the discovery of low abundant protein biomarkers for detection of early stage infections due to their poor mass sensitivity. Therefore methods for enhancing the detection sensitivity of labeled fragments are in critical demand.[60] To determine the ionization efficiency and mass sensitivity enhancement capability of HAT probe **1o** in comparison with free peptide, sulfone probe **1i** and the **IAA** probe, we carried out MS of the mixture of **1o**-labeled peptide Ac-GCF (5 μM) and **1i**-labeled peptide Ac-GCF (5 μM), **1o** labeled peptide Ac-GCF (5 μM) and **IAA**-labeled peptide Ac-GCF (5 μM) and **1o** labeled peptide Ac-GCF (5 μM) and unlabeled peptide Ac-GCF (5 μM) in water in equal concentrations. The **1o** tag on a peptide Ac-GCF delivered the most significant signal enhancement (1o-Ac-GCF : 1i-Ac-GCF, 100 : 5; 1o-Ac-GCF : IAA-Ac-GCF, 100 : 8; and 1o-Ac-GCF : Ac-GCF, 100 : 1.8) (ESI Fig. 24†). Next, a concentration assay confirmed that the tagged peptide 1o-Ac-GCF is detected up to 0.5 nanomolar concentration (ESI Fig. 24†).

HAT probe **N3-1o** also increased the detection sensitivity of the labeled protein fragments of the reduced insulin tremendously as compared to **IAA** as determined by MS without any purification (Fig. 5a and ESI Fig. 25†). For unlabeled and **IAA**-labeled reduced insulin fragments, very poor sensitivity was observed for both chains A and B of insulin. In fact chain A was undetectable by MS. **IAA** labelled only one free cysteine of chain B and **N3-1o** labeled both the free cysteines of chain B and all four free cysteines of chain A (Fig. 5a and ESI Fig. 25†). The HAT N3-1o-labeled fragments of reduced insulin showed significantly high mass intensities of both chains A and B. This also leads to the easy detection of chain A in MS that is otherwise undetectable and thus could be of high significance in proteomics studies.

HAT probes for gel-based ABPP studies

Finally, we turned our attention to the use of the HAT **N3-1o** reagent for activity-based protein profiling (ABPP) applications, owing to its high specificity and reactivity toward cysteine, as well as the small size of the HAT group that allows access to a broad range of proteins. To assess the proteome reactivity of this class of electrophiles, we evaluated the HAT probe **N3-1o** by gel-based competitive ABPP with NHS-tetramethylrhodamine (NHS-Rh) and iodoacetamide-tetramethylrhodamine (IA-Rh) using HEK293T cell lysate (Fig. 5b and c and ESI Fig. 26†). Blockade of IA-Rho labeling by pre-treatment with high concentrations of **N3-1o**, as indicated by the decreased in-gel fluorescence signal, is consistent with cysteine-labeling by **N3-1o** (Fig. 5b). The kinetic study of probes showed that **1o** and **N3-1o** are significantly more reactive than **IAA** (Fig. 2d), yet in the gel analysis experiment high amounts of **N3-1o** are required to achieve a similar labeling to **IAA**. This is because of the poor stability of the **N3-1o** cysteine conjugate products in the gel-based competitive ABPP experiments that required heating at 37 °C and 95 °C (Fig. 5b and ESI Fig. 19 and 26†). Supporting the specificity of **N3-1o** for cysteine labeling, **N3-1o** afforded no appreciable blockade of proteome labeling by the lysine-reactive probe NHS-Rh (Fig. 5c). These gel-based assays support that the high specificity of the HAT **N3-1o** probe observed for recombinant protein labeling extends to complex cell lysates (Fig. 5b and c and ESI Fig. 26†).

Conclusions

To close, HAT provides a unique and stable chemotype for chemoselective cysteine modification without cross reactivity with other amino acids. The reactivity of HAT is tuned by modulation of the type of aromatic ring, heteroatom on the aromatic ring, oxidation state and methylation states of the heteroatom. The resulting HAT probes are highly reactive towards cysteine and stable to hydrolysis. One of the unique features of HAT probes is their ability to be reversed easily by external stimuli to generate unmodified units in a traceless manner thus indicating the potential utility of this method in many research fields including protein immobilization, proteomics and current drug discovery efforts avoiding the permanent modification of proteins. Surprisingly, HAT probes enable reversal of the reactivity of nucleophilic cysteine to electrophilic dehydroalanine under mild basic conditions (pH 10.5) thus allowing for the modification of proteins at the cysteine site by various nucleophiles such as thiols and amines. Another unique feature about HAT probes is their ability to increase the mass sensitivity of the resulting bioconjugates by 100 fold leading to easy detection of cysteine conjugates in a complex mixture which is of high significance in proteomics studies for identification of low abundant protein fragments.

Finally, we anticipate that the selectivity of the HAT will enable future studies aimed at identifying and pharmacologically manipulating functional cysteines in whole proteomes, as well as a starting point for therapeutic interventions by reversible covalent inhibition of the reactive cysteines. These innovative HAT tools for probing cysteine would augment existing detection methods and will dramatically expand the toolbox for bioconjugation, proteome profiling, and targeting otherwise undruggable protein targets.

Data availability

The datasets supporting this article have been uploaded as part of the ESI.†

Author contributions

M. R. and K. T. designed the project. K. T. performed all the synthetic experiments and characterized the compounds by NMR and LCMS. K. T. synthesized and purified peptides for the experiments. S. M. M. and K. B. performed proteomic studies. All authors analyzed the results and contributed to writing the manuscript.

Conflicts of interest

There are no conflicts to declare.

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