It is of ongoing interest to develop new approaches for efficient and selective modification of cysteine residues on biomolecules. Here we present a comprehensive study on a newly developed isoxazolinium-mediated cysteine modification of peptides and proteins. Using a stoichiometric amount of isoxazolinium reagents generated in situ from a catalytic amount of silver salts, cysteine-containing peptides can be efficiently modified to afford products in nearly complete conversions. With the optimized conditions, free cysteine containing proteins HSA and BSA, as well as a site-directed mutated therapeutic protein (BCArg) can be efficiently and selectively labelled using small amounts of the isoxazolinium reagents. We find that the phenylacyl thioether linkage bearing an alkyne moiety can be rapidly cleaved under irradiation of UV-A light, giving the formation of a thioaldehyde moiety, which can be converted back to cysteine by reduction.
Selective modification of peptides and proteins has been recognized as an important tool for biological studies and drug development. Among the 20 natural amino acids, lysine and cysteine are prominent targets for chemical modification due to their high nucleophilicity. However, the prevalence of lysine residues on protein surface results in a difficulty to control the level and regioselectivity of the modification, and only a few examples of site-selective lysine labeling have been reported. In comparison, the low abundance (1.7%) and possible incorporation by site-directed mutagenesis allow cysteine to serve as an ideal residue for labeling. Conventional approaches for cysteine modification relied on α-haloacylboronyls (via S,S′2 reaction) and maleimides (via Michael addition). However, the relatively low chemoselectivity of α-haloacylboronies and potential hydrolysis of the maleimide-based conjugates prompted the development of a number of metal-free and transition metal-mediated cysteine modifications in the past decade by Davis, Bernardes, Pentelute, our group, and others. Despite these advances, it is of ongoing interest to develop new cysteine modification methods with high efficiency, excellent selectivity, and using easily accessible reagents under mild reaction conditions.

Development of bioconjugates with cleavable linkers has recently been recognized as an emerging area in chemical biology due to their versatile applications in drug development, proteomics, and in vivo imaging. Among the reported cysteine-selective modifications, only a few of them are cleavable, such as electron-deficient alkynes reported by our group, 5-methylene pyrrolones reported by Zhou, and 4-substituted cyclopentenones reported by Yin. However, all of those methods are of thiol-induced cleavage and the bioconjugates may undergo exchange reactions with thiols in plasma, limiting their applications in vivo studies. To overcome this limitation, an ideal approach is to develop a cysteine modification method utilizing a photocleavable and biologically compatible linker.

In 1978, Clark and Lowe reported that the phenylacetyl thioether linkage, formed by the reaction between cysteine and phenylacetyl bromide, underwent photolysis irradiated by UV light (λ_max = 342 nm) to form thioaldehyde and subsequently gave a chemically mutated serine residue through hydrolysis and chemical reduction. This is the first example of photocleavable cysteine modification. However, presumably due to the low selectivity of phenylacetic bromide reagents in cysteine modification, a long reaction time (3 h) for the UV-mediated photolysis and the lack of reliable technique to characterize the mutated residue, this method was rarely mentioned afterward. Dichlorotetrazine was reported as an efficient reagent for modification of peptides with two cysteine residues, giving stapled peptides with S,S-tetrazine linkages by Smith et al. Recently, the S,S-tetrazine linkage was uninstalled under irradiation of UV-B light (λ_max = 312 nm). However, the efficiency of cleavage under UV-A light (λ_max = 365 nm) was much lower, which might lead to the formation of side products. Recently, Bernardes et al. described that the isobutylenyl-caged thiols could be efficiently cleaved under mild UV irradiation in the presence of thiol sources and a photoinitiator. Besides, a UV-mediated photodestruction of genetic encoded ortho-nitrobenzyl (ONB)-caged cysteine on proteins has been reported by Chin et al. Apart from these examples, photocleavable cysteine modification still remains largely unexplored.

Using transition metal-based reagents for cysteine modifications has become attractive recently due to their high efficiency. However, employing a stoichiometric amount of organometallic reagents may lead to a relatively high content of transition metal-containing species as side products. In our previous works on modification of the N-terminal α-amino groups of peptides and proteins, we found that ketenes as intermediates generated in situ in manganese-catalyzed oxidative alkyne transformation were the key reagents for the modification. Inspired by this work, it is envisioned that the reactive intermediates generated in transition metal-catalyzed organic transformations can be utilized for the development of new cysteine modification reagents. Along this direction, we have reported that the electrophiic isoxazolinium ions, generated in silver-catalyzed transformations of propargylamine N-oxides, could be employed for efficient cysteine modification of a peptide and a protected cysteine model compound. The modification only required a catalytic amount of silver ions (25 mol%) to achieve a 99% conversion after 24 h.

In this paper, we report a comprehensive study on an efficient and highly chemoselective cysteine modification with a series of isoxazoliniums generated in situ via silver-catalyzed transformations of propargylamine N-oxides. The modification has been extended to free cysteine-containing peptides and proteins. The enzymatic activities as well as anticancer properties of a modified therapeutic protein (BCArg) have also been studied. In addition, by introduction of an alkyne moiety, the modified bioconjugates bearing phenylacetyl thioether linkages can be efficiently cleaved under irradiation of UV-A light (λ_max = 365 nm).

Results
Optimization studies. To prepare the isoxazolinium reagents, propargylamines were modularly synthesized via gold-catalyzed three-component coupling reactions of aldehydes, amines, and alkynes developed by Li and us (Supplementary Fig. 1). Then, by treatment with a stoichiometric amount of m-CPBA and a catalytic amount of AgNO3 sequentially, the propargylamines were stepwise converted to propargylamine N-oxides and isoxazoliniums (Supplementary Fig. 2). The in situ generated isoxazoliniums could be stabilized by hydrogen-bonding interactions in protic solvents and the reagents could be stored at −20 °C for repeated usage.

To begin our study, cysteine-containing peptide STSSCNLSK 1a and isoxazolinium reagent 2a were employed as model substrates for condition screening. By treatment of peptide STSSCNLSK 1a (0.1 mM) with isoxazolinium reagent 2a (1 equivalent) with 1 mol% of AgNO3 in PBS 7.4 buffer/CH3CN (19:1) at 25 °C for 2 h, following the mechanism depicted in Fig. 1e, modified peptide 3a was afforded in 79% conversion (Table 1, entry 1). Increasing the loading of AgNO3 from 1 to 2.5 and 5 mol% led to an improvement of the conversion to 86 and 96% (Entries 2–3). The total ion chromatogram of the modified mixtures (using conditions depicted in entry 3) by LC-MS analysis indicated the modification was efficient and clean (Fig. 2a). MS/MS analysis of the modified peptide 3a revealed that only the cysteine residue was modified, while other residues remained intact (Fig. 2b). However, further increasing the loading of AgNO3 to 10 mol% resulted in a significant drop of the conversion to 68%, which was attributed to the low stability of isoxazolinium ions with a high content of silver salts. Moreover, within 2 h, addition of excessive equivalents of the isoxazolinium reagent 2a (2–5 equivalents) gave modified peptide 3a in lower conversions (entries 5–8), though nearly complete conversions could be achieved after 24 h.

We next attempted to study the effects of pH values and temperature on the modification using time course experiments. Screening reactions in PBS buffer with different pH values indicated that the reaction could be conducted with good to excellent conversions (88–99%) from slightly acidic to basic media (pH 5.3 to 9.0), and basic conditions would further increase the conversion (entries 9–12 and Fig. 2c). This observation could be explained by the pKa value of the thiol group (~8.5) of cysteine. Basic conditions would promote the
deprotonation of the thiol group, leading to stronger nucleophilicity, which facilitated the modification. Time course experiments at different temperatures were also performed, suggesting that the reaction proceeded even faster at 37 °C, giving modified peptide 3a in >80% conversion after 15 min, while low conversion (<50%) was afforded after 4 h when the reaction was performed at 4 °C (entries 13–14 and Fig. 2d). Control experiments indicated that isoxazolinium ions were the key reagents for the modification (entries 15–16).

We also studied the compatibility of the modification under different aqueous solutions. With the optimized conditions (using 1 equivalent of isoxazolinium reagent 2a with 5 mol% of AgNO3), the modification proceeded smoothly under PBS, Tris-HCl, imidazole-HCl, citric acid-Na2HPO4, or NaCl medium, giving conversions in 88–97% (Supplementary Methods). These findings implied that this cysteine modification approach could proceed efficiently in various buffers at physiological pH (~7.4) requiring only a stoichiometric amount of isoxazolinium reagent and a catalytic amount (5 mol%) of silver salts.

We then sought to investigate the regioselectivity of the modification. Treatment of cysteine-containing peptides STSSSSCNLSK 1a, AYEMWCFSQR 1b, and KSTFC 1c with a stoichiometric amount of isoxazolinium reagent 2a gave modified peptide 3a, 4a, and 5a in 96, 99, and 99% conversion, respectively (Table 2, entries 1–3). MS/MS analysis revealed that only the cysteine residues on the peptides were modified, while other residues remained intact. Control experiments using peptides 1d–i without free cysteine residue resulted in no modification.

### Table 1 Optimization of modification conditions

| Entry | 2a (equiv.) | AgNO3 (mol%) | pH values | Temp. (°C) | Conversions (%) |
|-------|-------------|--------------|-----------|------------|----------------|
| 1     | 1           | 1            | 7.4       | 25         | 79             |
| 2     | 1           | 2.5          | 7.4       | 25         | 86             |
| 3     | 1           | 5            | 7.4       | 25         | 96             |
| 4     | 1           | 10           | 7.4       | 25         | 68             |
| 5     | 2           | 10           | 7.4       | 25         | 91             |
| 6     | 3           | 15           | 7.4       | 25         | 87             |
| 7     | 4           | 20           | 7.4       | 25         | 84             |
| 8     | 5           | 25           | 7.4       | 25         | 77             |
| 9     | 1           | 5            | 5.3       | 25         | 88             |
| 10    | 1           | 5            | 6.3       | 25         | 91             |
| 11    | 1           | 5            | 8.0       | 25         | 98             |
| 12    | 1           | 5            | 9.0       | 25         | 98             |
| 13    | 1           | 5            | 7.4       | 37         | 99             |
| 14    | 1           | 5            | 7.4       | 4          | 39             |
| 15    | 0           | 0            | 7.4       | 25         | 0              |
| 16    | 1d          | 0            | 7.4       | 25         | 0              |

*aConditions of the modifications: treatment of STSSSSCNLSK 1a (0.1 mM) with isoxazolinium reagent 2a (different amounts with different loadings of AgNO3) in 50 mM PBS buffer/CH3CN (19:1) with different pH values for 2 h. bConversion of the modification was determined by LC-MS analysis. cNearly complete conversion (≥99%) was achieved after 24 h. dReagent was prepared without addition of AgNO3.

Fig. 1 Reagents and strategy for cysteine modification. a Classical reagents used for cysteine modification. b Reagents used for thiol-induced cleavable cysteine modification. c Reagents used for photocleavable cysteine modification. d General strategy for cysteine modification using isoxazoliniums. e Reaction mechanism of cysteine modification with isoxazolinium reagents.
buffer/CH3CN (19:1) at 25 °C for 2 h. These substituents on R1 would lead to the formation of switchable xazolinium reagent 98 product pro

further screened the scope of R2 with various substituents. Table 3, entry 1). The conversion was lower (87%) when the six-membered ring gave the highest conversion in 96%

afforded by treatment of peptide STSSSCNLK 1a (0.1 mM) with isoxazolinium reagent 2a (1 equivalent) containing 5 mol% of AgNO3 in 50 mM PBS 7.4 buffer/CH3CN (19:1) at 25 °C for 2 h.

In MS/MS spectrum of cysteine-modified peptide 3a. c Time course experiments of the formation of cysteine-modified peptide 3a at different pH values. d Time course experiments of the formation of cysteine-modified peptide 3a at different temperatures (entries 4–9), suggesting that this modification was highly chemoselective toward the thiol moiety of the cysteine residue in the presence of other nucleophilic residues, such as N-terminus, lysine, histidine, tryptophan, and methionine, etc.

**Scope of the isoxazolinium reagent.** As propargylamines could be easily accessed by modular synthesis, we moved on to study the structure–reactivity relationship of the isoxazolinium reagents (Supplementary Fig. 3). Isoxazoliniums 2a–d bearing amine moieties with different ring sizes were first screened for the modifications. The results indicated that amine moiety with six-membered ring gave the highest conversion in 96% (Table 3, entry 1). The conversion was lower (87%) when the amine moiety with five-membered ring was utilized (entry 2). Moreover, conducting the modifications with amine moieties bearing larger ring sizes caused significantly drop of the conversions and the formation of β-thio-substituted ketone product 3aa via Michael addition (entries 3–4).12 Keeping the optimal six-membered ring size on the amine moiety, isoxazoliniums with different combinations of R1 were screened. Isoxazoliniums 2e–f with R1 bearing alkyl groups were well compatible with the modifications giving the formation of 3a in 98–99% conversions (entries 5–6). However, when isoxazolinium reagent 2g (R1 = aryl) was employed, 3a was afforded in 61% conversion, while α-thio-substituted enone product 3ab was given in 27% conversion via amine elimination (entry 7). When isoxazolinium reagent 2h (R1 = H) was used, only amine elimination product 3ac was obtained in 98% conversion (entry 8). These findings indicated that different substrates on R1 would lead to the formation of switchable product profiles. Since R2 was incorporated on the resulting bioconjugates while the amine moiety and R1 were cleaved, we further screened the scope of R2 with various substituents. Isoxazolinium reagents 2i–n with electron-donating groups (R2 = OCH3, OCH2CH3, CH3) and electron-withdrawing groups (R2 = F, Br, COCH3) were well tolerated with the modifications giving modified peptides 3b–g in 97–99% conversions (entries 9–14). By changing the benzene moiety to a naphthalene moiety, the formation of 3h was achieved in 99% (entry 15). Isoxazolinium reagents 2p–q bearing alkyl moieties, with potential applicability for sequential modifications using click reactions, were also conducted for the modifications, resulting in the formation of 3i–j in 97 and 98% conversions, respectively (entries 17–18).

To further demonstrate the utility of this cysteine modification, we attempted to expand the scope of this modification by using functional isoxazolinium reagents 2t–v (Fig. 3d). Employment of coumarin-derived isoxazolinium 2t (1.5 equivalents) and fluorescein-derived isoxazolinium 2u (2.5 equivalents) gave the corresponding modified peptides 3k in 84% conversion and 3l in 86% conversion. In addition, PEGylated peptide 3m could be afforded by using a stoichiometric amount of PEG-derived isoxazolinium 2v.

**Peptide stapling.** Peptide stapling with covalent linkages via macrocyclization reactions has been demonstrated to be an important strategy for constraining the peptide conformations, leading to a potential improvement of their proteolytic stability and cell permeability. However, the approaches for stapling peptides using native amino acids as handles still remained limited.16 With this efficient and selective cysteine modification using isoxazolinium reagents in hand, we synthesized bis-oxazolinium reagent 2u for macrocyclization of peptides YCKEACAL 1j and YCKEAGGACL 1k with two cysteine residues, respectively. Stapled products 3n (i, i + 4) and 3o (i, i + 7) were afforded in moderate-to-good conversion, indicating that the bis-isoxazoliniums were useful for the construction of covalently stapled peptides via macrocyclization (Fig. 3c).
Stability studies. Stability of the modified bioconjugates was evaluated by treatment of the modified peptide 3a with excessive thiol-containing reagents, reducing reagents and oxidizing reagents. Investigations were conducted by treatment of modified peptide 3a with 500 equivalents of L-cysteine, DL-homocysteine, glutathione (GSH), and dithiothreitol (DTT), respectively. After 3 h, LC-MS analysis of the resulting mixtures revealed that the modified peptide 3a still remained intact. Treatment of the

**Table 2 Investigation of the regioselectivity**

| Entry | Peptides | Conversions (%) |
|-------|----------|-----------------|
| 1     | STSSSCNLSK 1a | 96              |
| 2     | AYEMWCFSQR 1b | 99              |
| 3     | KSTFC 1c     | 99              |
| 4     | STSSSANLSK 1d | 0               |
| 5     | STSSSHNL5K 1e | 0               |
| 6     | AYEMWSFQR1f  | 0               |
| 7     | PSKFR 1g     | 0               |
| 8     | DSKFR 1h     | 0               |
| 9     | QSKFR 1i     | 0               |

*aConditions of the modifications: treatment of peptides 1a–i (0.1 mM) with isoxazolinium reagent 2a (1 equivalent with 5 mol% of AgNO3) in 50 mM pH 7.4 PBS buffer/CH3CN (19:1) at 25 °C for 2 h.

*bConversion of the modification was determined by LC-MS analysis.

**Table 3 Investigation of the scope of isoxazoliniums**

| Entry | Isoxazolinium reagents | Modified peptides | Conversions (%) |
|-------|------------------------|-------------------|-----------------|
| 1     | 2a                     | 3a, 3aa           | 96              |
| 2     | 2b                     | 3a                | 87              |
| 3     | 2c                     | 3a, 3aa           | 31, 21          |
| 4     | 2d                     | 3a, 3aa           | 27, 20          |
| 5     | 2e                     | 3a                | >99             |
| 6     | 2f                     | 3a                | 98              |
| 7     | 2g                     | 3a, 3ab           | 61, 27          |
| 8     | 2h                     | 3a, 3ac           | 98              |
| 9     | 2i                     | 3b                | 97              |
| 10    | 2j                     | 3c                | 98              |
| 11    | 2k                     | 3d                | 97              |
| 12    | 2l                     | 3e                | 97              |
| 13    | 2m                     | 3f                | 99              |
| 14    | 2n                     | 3g                | 98              |
| 15    | 2o                     | 3h                | 99              |
| 16    | 2p                     | 3i                | 97              |
| 17    | 2q                     | 3j                | 98              |

*aConditions of the modifications: treatment of STSSSCNLSK 1a (0.1 mM) with isoxazolinium reagent 2a–q (1 equivalent with 5 mol% of AgNO3) in 50 mM pH 7.4 PBS buffer/CH3CN (19:1) at 25 °C for 2 h.

*bConversion of the modification was determined by LC-MS analysis.
modified peptide 3a with 500 equivalents of common reducing reagents, TCEP as well as sodium ascorbate also led to no interference with the modified bioconjugates. These findings implied that the phenylacyl thioether linkage formed after the modification was stable toward environments with thioc-containing reagents and common reducing reagents. H2O2 as an oxidizing reagent was also examined. Treatment of 500 equivalents of H2O2 with modified peptide 3a oxidized the thioether moiety to the corresponding sulfoxide moiety as confirmed by LC-MS/MS analysis. Under the same conditions, using 500 equivalents of Oxone (potassium peroxymonosulfate) as oxidant, the thioether moiety on the modified peptide 3a was further oxidized to the corresponding sulfone moiety. These findings implied that the stability of the thioether linkage toward oxidants was consistent to that on methionine residue, which was previously reported method using electron-deficient alkynes (Supplementary Fig. 4).15 Treated with 1 equivalent of 2-bromoacetonone 6, 4a isoxazolinium reagent 1b was converted to modified peptide 4a in 99% conversion which was comparable with modification using isoxazolinium reagent 2a (99% conversion as depicted in Table 3, entry 2). However, if 5 equivalents of 6 was employed, di-modified peptide 4aa with a second modification on methionine residue was afforded in 10% conversion after 2 h. After 3 days, di-modified peptide 4aa conversion was increased to 53%. In contrast, by treatment of 1b with 5 equivalents of isoxazolinium reagent 2a for 3 days, apart from cysteine, no other residues were modified, suggesting that the isoxazolinium reagent was highly chemoselective for cysteine modification. A mixture of 1 equivalent of N-benzylmaleimide 7 with 4a isoxazolinium reagent 1b gave modified peptide 4b in 99% conversion in 2 h. After 3 days, it was found that 52% hydrolyzed derivative 4ba was afforded. Under the same conditions, modified product 4a afforded using isoxazolinium reagent 2a still remained intact. Treatment of 4a isoxazolinium reagent 1b with 1 equivalent of electron-deficient alkyne 1-phenyl-2-propyn-1-one 8 gave modified peptide 4c in 99% conversion in 2 h. By addition of excess thiol-containing reagent L-cysteine (50 equivalents), cleavage product 1b was afforded in 30%, which was consistent with our previous observation that the vinyl sulfide linkage could be cleaved by addition of excess thiol-containing reagents.15 In contrast, modified product 4a afforded through the modification using isoxazolinium reagent 2a still remained intact, supporting the aforementioned results that the phenylacyl thioether linkage formed was stable toward excess thiols.

**Application to protein modification.** After a comprehensive study on the efficiency, chemoselectivity, scope, and stability of this isoxazolinium-mediated cysteine modification, we further explored its applicability for protein bioconjugation. Bovine serum albumin (BSA) and human serum albumin (HSA) with a single free cysteine residue were utilized for bioconjugation. Treatment of HSA or BSA (0.1 mM) with isoxazolinium reagent 2a (1 equivalent) with 5 mol% of AgNO3 in PBS 7.4 buffer/CH3CN (19:1) at 25 °C for 2 h afforded modified protein HSA-1 in 84% or BSA-1 in 94% conversion by LC-MS analysis (Fig. 3c-e). Upon trypsin digestion, the modification was found on Cys34 residue on peptide fragment GLVLIAFSQYLQQCPFDEHVK of HSA-1 or ALVLIAFSYGLQQCPFDEHVK of BSA-1, while other residues still remained intact. For non-cysteine-containing proteins, insulin, RNaseA, and lysozyme, under the same reaction conditions, no modification was found. These results indicated that the isoxazolinium-mediated modification could be conducted with high efficiency and chemoselectivity in protein modification.

Arginase is a family of enzymes that converts L-arginine to L-ornithine. By arginine depletion, arginase has been investigated to possess anticancer effects toward a broad spectrum of cancer types.48 The first-generation non-site-specific lysine PEGylated human arginase I with a prolonged circulating half-life is undergoing phase II clinical trials.49 Batillus Caldovelox arginase (BCArg) is a type of arginase with high production yields, and it can be simply purified.50 We attempted to proceed a site-specific modification of BCArg with our newly developed cysteine modification using isoxazolinium reagents. A free cysteine residue was mutated on Ser161 via site-directed mutagenesis (Supplementary Fig. 5). Treatment of the mutated BCArg (0.1 mM) with isoxazolinium reagent 2a (2.5 equivalents) with 5 mol% of AgNO3 in Tris-HCl 7.4 buffer/CH3CN (19:1) at 25 °C for 2 h
afforded modified protein BCArg-1 in 83% conversion (Fig. 4c). Upon trypsin digestion, the modification was found on Cys161 residue on peptide fragment LQVIWYDAHG-DVNTAETSPGNHGMPLAASLGFGHPALTQIGGYCPK of BCArg-1. Circular dichroism (CD) measurement of BCArg and BCArg-1 revealed that the secondary structures retained after the modification (Supplementary Fig. 6, Supplementary Table 1). In addition, as determined by ICP-MS analysis, only 1.8 mol% of the silver content was found from the modiﬁed protein BCArg-1.

Circular dichroism (CD) measurement of the modiﬁed proteins, the CD spectra of native and modiﬁed proteins are shown in Supplementary Table 2. The CD spectra of the modiﬁed proteins BCArg-1 and BCArg-2 were found to be comparable with BCArg (Table 4). The anticancer properties of the native and modiﬁed BCArg were examined using a breast cancer cell line MDA-MB-231 and a lung cancer cell line NCI-H23 (Supplementary Table 7). The IC50 values measured indicated that the antitumor efﬁcacy is comparable with the native BCArg.

Table 4 Enzymatic activities of native and modiﬁed BCArg

| Sample | BCArg | BCArg-1 | BCArg-2 |
|--------|-------|---------|---------|
| Specific activity (U/mg) | 109.77 ± 4.95 | 98.41 ± 13.95 | 99.64 ± 6.11 |
| **IC50** values for MDA-MB-231 (U/mL) | 3.587 ± 1.278 | 4.719 ± 1.438 | 3.144 ± 1.232 |
| **IC50** values for NCI-H23 (U/mL) | 3.921 ± 1.318 | 5.295 ± 1.262 | 5.146 ± 1.475 |

Using silver, the modiﬁed proteins showed a 2-fold increase in IC50 values compared to the native protein. The IC50 values of BCArg-1 and BCArg-2 were 3.921 U/mL and 5.295 U/mL, respectively. The CD spectra of the modiﬁed proteins BCArg-1 and BCArg-2 were found to be comparable with BCArg (Table 4). The anticancer properties of the native and modiﬁed BCArg were examined using a breast cancer cell line MDA-MB-231 and a lung cancer cell line NCI-H23 (Supplementary Table 7). The IC50 values measured indicated that the antitumor efﬁcacy is comparable with the native BCArg.

Photolysis studies. Finally, the photocleavable properties of the modiﬁed peptides and proteins were investigated (Fig. 5; Supplementary Table 6). Under irradiation of UV-A light (λmax = 365 nm) for 15 min, the protein-modiﬁed peptide 3a was converted to thioaldehyde product 10 in 41% conversion, while the
phenylacyl moiety was cleaved via a Norrish type II photolysis reaction (Supplementary Fig. 11). By introduction of an alkyn handle, modified peptide 3i could be cleaved more efficiently, leading to the formation of 10 in >99% conversion in 15 min. We monitored the photolysis reaction using time course experiments (Supplementary Fig. 12, 13). The results revealed that >90% of peptide 3i was cleaved in 10 min, while only 24% of peptide 3a was converted in the same period. Control reaction in the dark gave no photolysis of 3i. Concluding the reaction in a higher concentration of peptide 3i gave little influence on the efficiency of the photolysis reaction. Interestingly, by treatment of the cleavage peptide 10 in solution with NaBH₄ (50 mM) for 30 min, the peptide 10 could be reduced to give native peptide 1a in 97% conversion. We also investigated the photolysis using fluorescent labeled proteins HSA-3 and BSA-3. Under irradiation for 30 min, >60% of the linkages on the proteins were cleaved.

Discussion

In summary, we have presented a comprehensive study on a newly developed cysteine modification using isoxazoliums generated in situ via silver catalysis. The modification could proceed efficiently with high chemoselectivity toward cysteine residue on peptides and proteins. In most cases, only a stoichiometric amount of isoxazolium reagents with a catalytic amount of silver salts were needed to give high-to-excellent conversions. Besides, easily accessible isoxazolium reagents with versatile functional groups were compatible with this modification. Fluorescent tags could be efficiently labeled on the cysteine-containing peptides and proteins by directly employing fluorescent tag-derived isoxazolium reagents or by sequential modification using the azide-alkyne click reaction. The resulting phenylacyl thioether linkage was stable toward various thiol-containing reagents and reductants.

Investigation of the effect of the modification on a therapeutic protein (BCArg) revealed that the incorporated tag had little influence on the enzymatic activity and anticancer property of the protein, which suggested that the isoxazolium reagents could be potentially employed as promising reagents for labeling bioactive proteins in the future.

We have also found that incorporation of an alkyn moiety on the phenylacyl thioether linkage would induce a rapid photolysis under irradiation of UV-A light (λ max = 365 nm), and the resulting thioaldehyde moiety could be reduced to be thiol moiety rapidly. To the best of our knowledge, this is the first time to use LC-MS/MS analysis to monitor the formation of thioaldehyde by photolysis of phenylacyl thioether linkage in a peptide sample. Ongoing interest is to employ this photo cleavable cysteine modification for applications on proteomic analysis and drug development.

Methods

Synthesis and characterization. The synthetic procedures and characterization for compounds are depicted in Supplementary Methods, and references are listed in Supplementary Table 7. Chromatography and mass spectrometry data are presented in Supplementary Fig. 14–163. NMR spectra are presented in Supplementary Fig. 164–176.

Preparation of isoxazolium reagents 2a–u. For preparation of the isoxazolinium reagents 2a–t, a mixture of propargylamines 1a–t (0.05 mmol, 1 equiv.) and meta-chloroperoxybenzoic acid (m-CPBA, 0.05 mmol, 1 equiv.) was conducted in a solution of CH₃CN (0.50 mL) and H₂O (0.40 mL) at 25°C for 15 min, giving the formation of propargylamine N-oxides 11a–t in situ. After that, 0.10 mL of AgNO₃ solution (25 mM in H₂O) was added to the mixture and the reaction was continued at 25°C for 2 h to afford the isoxazolinium reagents 2a–t (5.25 MM in CH₃CN/H₂O (1:1)) at 1:1. The reagents were further diluted to 5 mM in CH₃CN/H₂O (1:1) and stored at −20°C for repeated usage. For preparation of isoxazolium reagents 2u, the mixture of propargylamine 1u, m-CPBA, and AgNO₃ was treated using the aforementioned conditions except for two equivalents of m-CPBA was used.

Modification of peptides using isoxazolium reagents 2a–t. A mixture of 10 µL of peptides 1a–t (1 mM in H₂O), 2 µL of isoxazolium reagents 2a–t (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 4 µL of CH₃CN, 4 µL of H₂O, and 80 µL of 50 mM pH 7.4 PBS buffer was treated in a 1.5 mL Eppendorf tube at 25°C for 2 h. The modified product was characterized by LC-MS and LC-MS/MS analysis. For modification of cysteine-containing peptide 1a with coumarin-derived isoxazolium reagent 2r, the modification was conducted using the aforementioned conditions except that 3 µL of isoxazolium reagents 2r (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 3.5 µL of CH₃CN, and 3.5 µL of H₂O were added. For modification of cysteine-containing peptide 1a with fluorescein-derived isoxazolium reagent 2s, the modification was conducted using the aforementioned conditions except that 5 µl of isoxazolium reagents 2s (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 2.5 µL of CH₃CN and 2.5 µl of H₂O were added.

Macrocyclization of peptides using isoxazolium reagents 2a. A mixture of 10 µl of peptides 1j–k (1 mM in H₂O), 2 µL of isoxazolium reagents 2a (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 4 µL of TCEP (12.5 mM in H₂O), 4 µL of CH₃CN, and 80 µL of 50 mM pH 7.4 PBS buffer was treated in a 1.5 mL Eppendorf tube at 25°C for 2 h. The modified product was characterized by LC-MS analysis.

Time course studies on the modification of peptide STSSSCLNLK 1a. A mixture of 20 µl of peptides 1a (1 mM in H₂O), 4 µl of isoxazolium reagent 2a (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 8 µL of CH₃CN, 8 µL of H₂O, and 160 µL of 50 mM PBS buffer with different pH values was treated in a 1.5 mL Eppendorf tube at different temperatures for 0–4 h. At each time point, the resulting mixture was collected and mixed with 10 µL of L-cysteine (50 mM in H₂O) to quench the modification. The resulting mixture was characterized by LC-MS and LC-MS/MS analysis to determine the conversion.

Modification of proteins using isoxazolium reagents 2a and 2p. A mixture of 10 µl of proteins (HSA, BSA, insulin, RNAsae, or lysozyme) (1 mM in 50 mM pH 7.4 PBS buffer), 2 µL of isoxazolium reagents 2a or 2p (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 4 µL of CH₃CN, 4 µL of H₂O, and 80 µL of 50 mM pH 7.4 PBS buffer was treated in a 1.5 mL Eppendorf tube at 25°C for 2 h. The modified product was characterized by LC-MS analysis. For modification of the therapeutic protein BCArg, a mixture of 100 µl of BCArg (1 mM in 50 mM pH 7.4 Tris-HCl buffer), 50 µL of isoxazolium reagents 2a or 2p (5 mM in CH₃CN/H₂O containing 5 mol% AgNO₃), 25 µL of CH₃CN, 25 µL of H₂O, and 800 µL of 50 mM pH 7.4 Tris-HCl buffer was treated in a 1.5 mL Eppendorf tube at 25°C for 2 h. The modified product was characterized by LC-MS analysis. Before sequential modification and analysis of biological properties, the modified proteins in solution were added into the filter of a Millipore Amicon™ Ultra-4 or ~10 -K centrifugal device. After that, the filter was filled with 15% CH₃CN/50 mM pH 7.4 PBS buffer (or Tris-HCl buffer in consistent to the buffer used in the modification). The Amicon™ Ultra device was centrifuged under 4000 RPM for 20 min by a BOECO CENTRIFUGE C-28A bench-top centrifuge. The purification process was repeated for three times. The 50 mM pH 7.4 PBS buffer was used instead of 15% acetonitrile/50 mM pH 7.4 PBS buffer at the last time. Modified proteins (0.1 mM) in 50 mM pH 7.4 PBS buffer were collected.

Sequential modification of proteins via Huisgen azide-alkyne cycloaddition. A mixture of 50 µl of alkynyl-functionalized proteins HSA-2 or BSA-2 (0.1 mM in 50 mM pH 7.4 PBS buffer), 5 µl of rhodamine azide 9 (5 mM in DMSO), 5 µl of TBTG (5 mM in DMSO), 5 µl of TCEP (5 mM in H₂O), 5 µl of CuSO₄ solution (5 mM in H₂O), and 30 µl of pH 7.4 PBS buffer was treated in a 1.5 mL Eppendorf tube at 25°C for 1 h. The modified product was characterized by LC-MS analysis. For modification of alkynyl-functionalized proteins BCArg-2, 50 mM pH 7.4 Tris-HCl buffer was used instead of PBS buffer.

Procedure for photo cleavage of modified peptide and proteins. Photolysis experiments were performed using a MAXIMA™ ML-3500S/FB Ultra-High Intensity UV-A Lamp (365 nm, 230 V, 50 Hz, 0.75 A/M). For photo cleavage of modified peptides, a mixture of 50 µl of modified peptides 3a or 3i (0.1 mM in 50 mM pH 7.4 PBS buffer/CH₃CN (19:1)), and 950 µl of 50 mM pH 7.4 PBS buffer
in a well of the Thermo Scientific Nunc® Cell-Culture Treated 24-well plate was irradiated by a UV-A Lamp (λexc = 365 nm) on an ice bath for 0–40 min. The resulting mixture was characterized by LC-MS and LC-MS/MS analysis. For time course experiments, at each time point, 20 μL of the mixture was collected for LC-MS analysis. For photocleavage of modified proteins, a mixture of 250 μL of modified proteins HSA-3 or BSA-3 (0.1 mM in 50 mM pH 7.4 PBS buffer/CH3CN (1:1)) and 750 μL of 50 mM pH 7.4 PBS buffer in a well of the Thermo Scientific Nunc® Cell-Culture Treated 24-well plate was irradiated by a UV-A Lamp (λexc = 365 nm) on an ice bath for 30 min. The resulting mixture was characterized by LC-MS analysis.

**Data availability**

All principal data with detailed experimental procedure and characterization of this work are included in this article, and its Supplementary Information or are available from the corresponding author upon reasonable request.

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Author contributions
M.-K.W. and Y.-C.L. conceptualized and supervised the study. J.-R.D., B.Y., J.-F.C. and K.-W.L. performed the organic synthesis in this work. J.-R.D., W.-M.Y. and K. K.-Y.K. performed the bioconjugation experiments and mass spectroscopy analysis of this work. S.-F.C., A.S.-L.L. and M.-C.C. purified and measured the biological activities of the anticancer proteins. Z.Z. performed the SDS-PAGE analysis. J.-R.D. performed the photolysis experiments. M.-K.W., Y.-C.L., J.-R. D. and W.-M.Y. prepared this paper.

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