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Chemoselective and photocleavable cysteine modification of peptides and proteins using isoxazoliniums

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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.

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Selective modification of peptides and proteins has been recognized as an important tool for biological studies and drug development[1–6]. 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[1,7–10]. In comparison, the low abundance (1.7%) and possible incorporation by site-directed mutagenesis allow cysteine to serve as an ideal residue for labeling[11,12]. Conventional approaches for cysteine modification relied on α-halocarbonyls (via S_N2 reaction) and maleimides (via Michael addition). However, the relatively low chemoselectivity of α-halocarbonyls 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[13–30]. Despite those 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[31]. Among the reported cysteine-selective modifications, only a few of them are cleavable, such as electron-deficient alkynes reported by our group[15], 5-methylene pyrrolones reported by Zhou[24], and 4-substituted cyclopentenones reported by Yin[25]. 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 in vivo studies[32,33]. To overcome this limitation, an ideal approach is to develop a cysteine modification method utilizing a photocleavable and biologically compatible linkage[31].

In 1978, Clark and Lowe reported that the phenylacyl thioether linkage, formed by the reaction between cysteine and phenylacyl 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[34,35]. This is the first example of photocleavable cysteine modification. However, presumably due to the low selectivity of phenylacyl 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.[21]. The *S*,*S*-tetrazine linkage was unstapled 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[21]. Recently, Bernardes et al. described that the isobutylene-caged thiols could be efficiently cleaved under mild UV irradiation in the presence of thiol sources and a photoinitiator[36]. Besides, a UV-mediated photodeprotection of genetic encoded *ortho*-nitrobenzyl (ONB)-caged cysteine on proteins has been reported by Chin et al.[37]. 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[28–30]. 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[38,39]. 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 electrophilic 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%) [40].

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 phenylacyl 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)[41–44]. Then, by treatment with a stoichiometric amount of *m*-CPBA and a catalytic amount of $AgNO_3$ sequentially, the propargylamines were stepwise converted to propargylamine *N*-oxides and isoxazoliniums (Supplementary Fig. 2)[40]. The in situ generated isoxazoliniums could be stabilized by hydrogen-bonding interactions in protic solvents[45], and the reagents could be stored at −20 °C for repeated usage.

To begin our study, cysteine-containing peptide STSSSCNLSK **1a** and isoxazolinium reagent **2a** were employed as model substrates for condition screening. By treatment of peptide STSSSCNLSK **1a** (0.1 mM) with isoxazolinium reagent **2a** (1 equivalent) with 1 mol% of $AgNO_3$ in PBS 7.4 buffer/CH_3CN (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 $AgNO_3$ 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 $AgNO_3$ 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

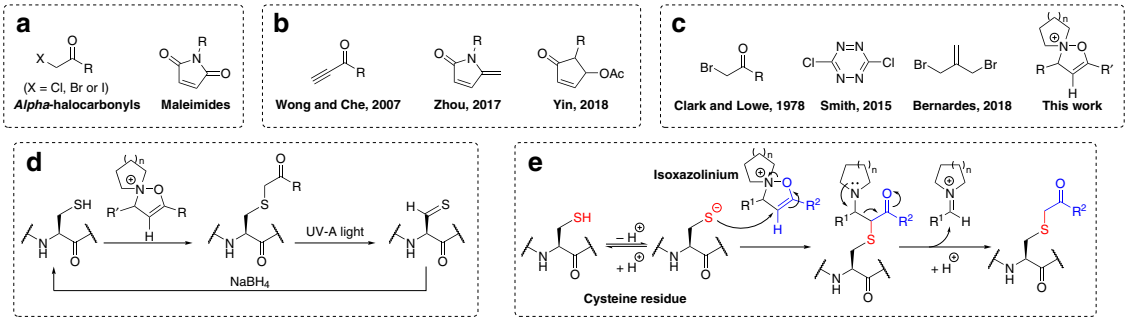
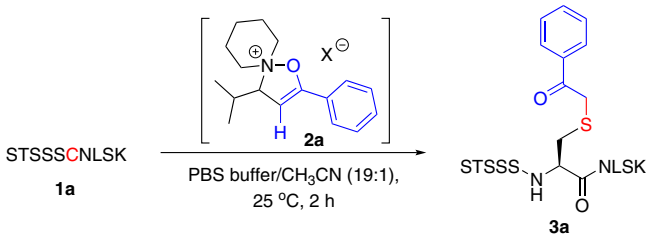


Fig. 1 Reagents and strategy for cysteine modification. **a** Classical reagents used for cysteine modification. **b** Reagents used for thiol-induced cleavable cysteine modification. **c** Reagents used for photocleavable cysteine modification. **d** General strategy for cysteine modification using isoxazoliniums. **e** Reaction mechanism of cysteine modification with isoxazolinium reagents

Table 1 Optimization of modification conditions[a]



Entry	2a (equiv.)	$AgNO_3$ (mol%)	pH values	Temp. (° C)	Conversions (%)[b]
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[c]
6	3	15	7.4	25	87[c]
7	4	20	7.4	25	84[c]
8	5	25	7.4	25	77[c]
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	99
13	1	5	7.4	37	99
14	1	5	7.4	4	39
15	0	0	7.4	25	0
16	1[d]	0	7.4	25	0

[a]Conditions of the modifications: treatment of STSSSCNLSK **1a** (0.1 mM) with isoxazolinium reagent **2a** (different amounts with different loadings of $AgNO_3$) in 50 mM PBS buffer/CH_3CN (19:1) with different pH values for 2 h. [b]Conversion of the modification was determined by LC-MS analysis. [c]Nearly complete conversion (≥99%) was achieved after 24 h. [d]Reagent was prepared without addition of $AgNO_3$

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 $AgNO_3$), the modification proceeded smoothly under PBS, Tris-HCl, imidazole-HCl, citric acid–Na_2HPO_4, 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 STSSSCNLSK **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

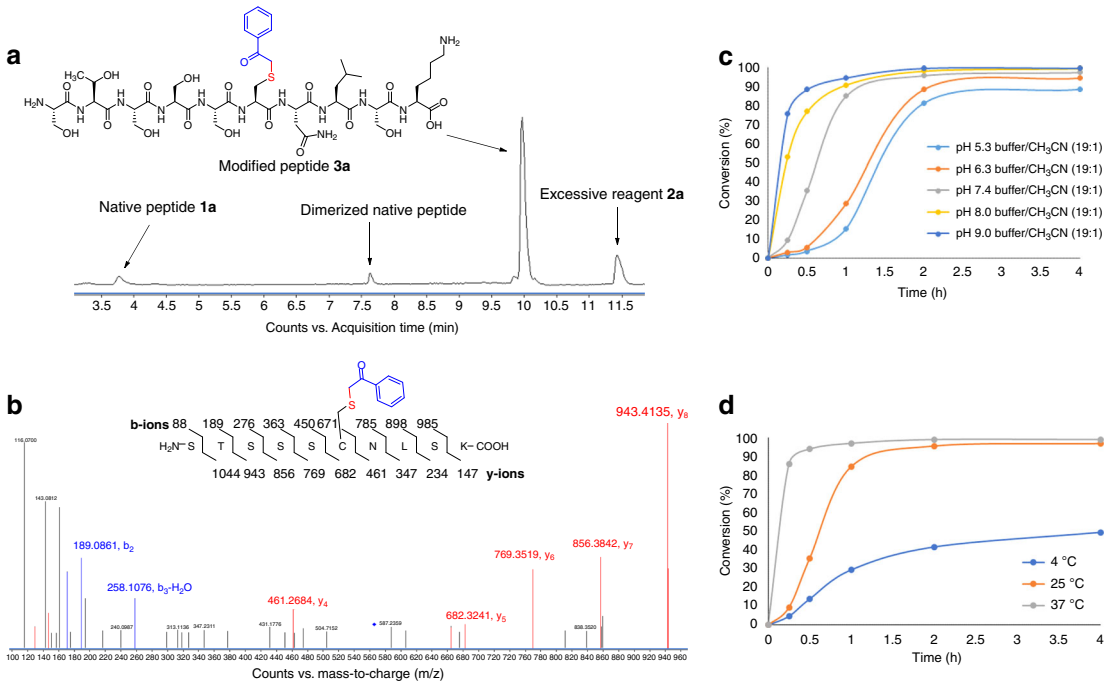


Fig. 2 Modification of peptide STSSSCNLSK **1a** with isoxazolinium reagent **2a**. **a** The total ion chromatogram by LC-MS analysis of the modified mixtures, afforded by treatment of peptide STSSSCNLSK **1a** (0.1 mM) with isoxazolinium reagent **2a** (1 equivalent) containing 5 mol% of $AgNO_3$ in 50 mM PBS 7.4 buffer/CH_3CN (19:1) at 25 °C for 2 h. **b** 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

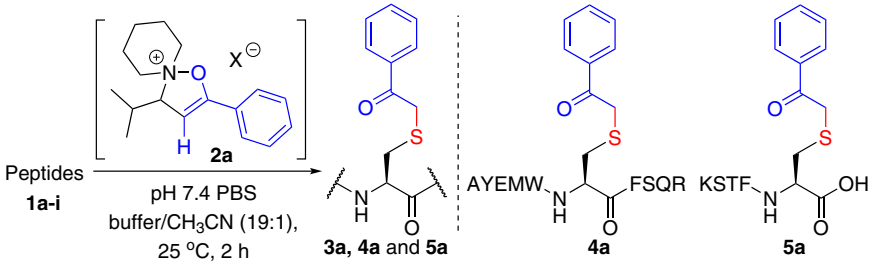
(entries 4–9), suggesting that this modification was highly chemoselective toward the thiol moiety of the cysteine residue in the presence of other nucleophilic residues, such as N-terminus, lysine, histidine, tryptophan, and methionine, etc.

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 R^1 were screened. Isoxazoliniums **2e–f** with R^1 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** (R^1 = 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** (R^1 = H) was used, only amine elimination product **3ac** was obtained in 98% conversion (entry 8). These findings indicated that different substituents on R^1 would lead to the formation of switchable product profiles. Since R^2 was incorporated on the resulting bioconjugates while the amine moiety and R^1 were cleaved, we further screened the scope of R^2 with various substituents. Isoxazolinium reagents **2i–n** with electron-donating groups ($R^2 = OCH_3$, OCH_2CH_3, CH_3) and electron-withdrawing groups (R^2 = F, Br, $COCH_3$) 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 alkyne 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[46]. With this efficient and selective cysteine modification using isoxazolinium reagents in hand, we synthesized bis-isoxazolinium 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).

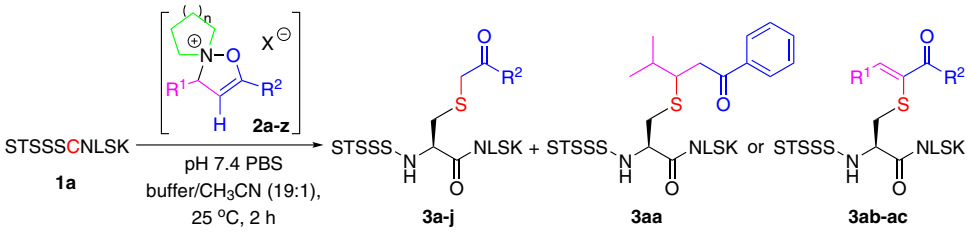
Table 2 Investigation of the regioselectivity[a]



Entry	Peptides	Conversions (%)[b]
1	STSSSCNLSK **1a**	96
2	AYEMWCFSQR **1b**	99
3	KSTFC **1c**	99
4	STSSSANLSK **1d**	0
5	STSSSHNLSK **1e**	0
6	AYEMWSFHQR **1f**	0
7	PSKFR **1g**	0
8	DSKFR **1h**	0
9	QSKFR **1i**	0

[a]Conditions of the modifications: treatment of peptides **1a-i** (0.1 mM) with isoxazolinium reagent **2a** (1 equivalent with 5 mol% of $AgNO_3$) in 50 mM pH 7.4 PBS buffer/CH_3CN (19:1) at 25 °C for 2 h.
[b]Conversion of the modification was determined by LC-MS analysis

Table 3 Investigation of the scope of isoxazoliniums[a]



Entry	Isoxazolinium reagents	Modified peptides	Conversions (%)[b]
1	**2a**	**3a**	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**	**3ac**	98
9	**2i**	**3b**	97
10	**2j**	**3c**	98
13	**2k**	**3d**	97
14	**2l**	**3e**	97
15	**2m**	**3f**	99
16	**2n**	**3g**	98
17	**2o**	**3h**	99
18	**2p**	**3i**	97
19	**2q**	**3j**	98

[a]Conditions of the modifications: treatment of STSSSCNLSK **1a** (0.1 mM) with isoxazolinium reagent **2a–q** (1 equivalent with 5 mol% of $AgNO_3$) in 50 mM pH 7.4 PBS buffer/CH_3CN (19:1) at 25 °C for 2 h. [b]Conversion of the modification was determined by LC-MS analysis

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

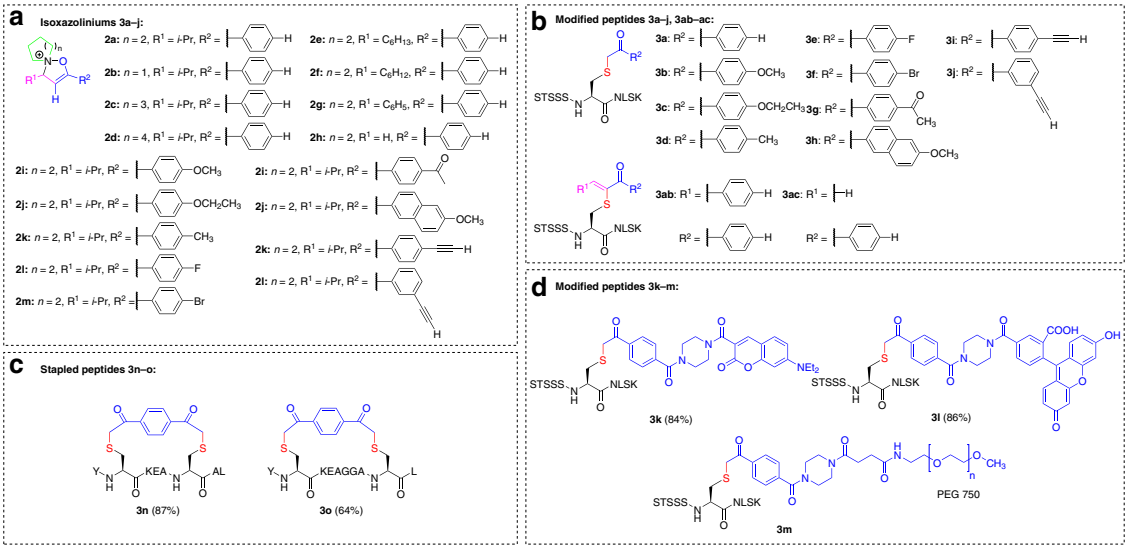


Fig. 3 Scope of the isoxazolinium reagents and modified peptides. **a** Scope of the isoxazolinium reagents **3a–j**. **b** Scope of the modified peptides **3a–j** and **3ab–ac**. **c** scope of the stapled peptides **3n-o** (conversion). **d** Scope of the modified peptides **3k–m** (conversion) with functional tags

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 thiol-containing reagents and common reducing reagents. H_2O_2 as an oxidizing reagent was also examined. Treatment of 500 equivalents of H_2O_2 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 reported to be oxidized by addition of oxidizing reagents[47].

To provide more insights on this newly developed cysteine modification, we compared this method with the conventional approaches using α-halocarbonyls and maleimides, and a previously reported method using electron-deficient alkynes (Supplementary Fig. 4)[15]. Treated with 1 equivalent of 2-bromoacetophenone **6**, AYEMWCFSQR **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 AYEMWCFSQR **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 AYEMWCFSQR **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 excessive 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 $AgNO_3$ in PBS 7.4 buffer/CH_3CN (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 GLVLIAFSQYLQQCPF-DEHVK of **HSA-1** or ALVLIAFSQYLQQCPFDEHVK 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]. *Bacillus 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 with isoxazolinium reagent **2a** (2.5 equivalents) with 5 mol% of $AgNO_3$ in Tris-HCl 7.4 buffer/CH_3CN (19:1) at 25 °C for 2 h

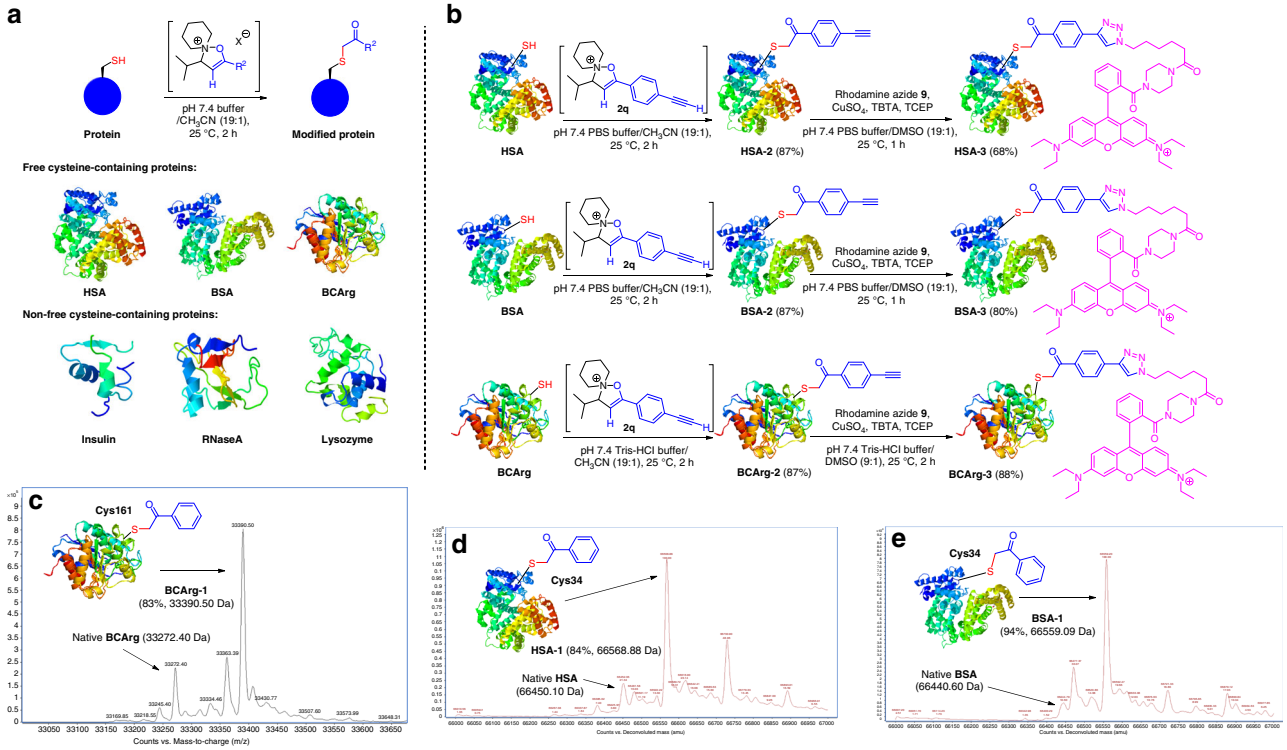


Fig. 4 Modification of proteins with isoxazolinium reagents. **a** General scheme of modification of proteins with (or without) free cysteine residues. **b** Fluorescent labeling of proteins. **c** Mass deconvolution spectrum of **HSA-1** (84% conversion). **d** Mass deconvolution spectrum of **BSA-1** (94% conversion). **e** Mass deconvolution spectrum of **BCArg-1** (83% conversion)

afforded modified protein **BCArg-1** in 83% conversion (Fig. 4c). Upon trypsin digestion, the modification was found on Cys161 residue on peptide fragment LGVIWYDAHG-DVNTAETSPSGNIHGMPLAASLGFGHPALTQIGGYCPK 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 **BCArg-1** sample after a filtration, suggesting it would be promising to examine the enzyme activities and the antitumor efficiencies of the modified proteins (Supplementary Tables 2–4). Using isoxazolinium reagent **2p**, an alkyne handle was incorporated on **BCArg** to give the formation of alkyne-functionalized **BCArg-2** in 87% conversion. After washing out the excessive reagents, the enzymatic activities of the resulting 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 Fig. 7, 8). The IC_{50} values measured indicated that the antitumor efficacies of the modified proteins **BCArg-1** and **BCArg-2** were similar to that of **BCArg** (Supplementary Table 5). These findings revealed that the therapeutic proteins still retained their anticancer properties after the modification.

Labeling of proteins with fluorescent tags provides an opportunity for convenient protein staining in SDS-PAGE gel using fluorescent analysis, and also has potential for in vivo tracking the uptake and physiological parameters[5]. As the isoxazolinium-mediated cysteine modification could be successfully applied on modification of proteins, we next demonstrated the utility of this reaction for fluorescent labeling of proteins. Using isoxazolinium reagent **2p**, alkyne handles were first incorporated on the free cysteine residues of **HSA**, **BSA**, and **BCArg** with good conversions. After washing out the excessive reagents, the alkyne-functionalized proteins (**HSA-2**, **BSA-2**, and **BCArg-2**) were rapidly labeled with red fluorescent rhodamine dyes with good conversions by azide-alkyne Huisgen cycloadditions (Fig. 4b). SDS-PAGE analysis revealed that, rhodamine-labeled proteins (**HSA-3**, **BSA-3**, and **BCArg-3**) were observed using fluorescent analysis, while no fluorescent signal was observed for native proteins (**HSA**, **BSA**, and **BCArg**) or alkyne-functionalized proteins (**HSA-2**, **BSA-2**, and **BCArg-2**). Employing Coomassie blue staining on the same gel, deep blue color signals of native, alkyne-functionalized as well as rhodamine-labeled proteins were observed, indicating that the fluorescent tags have been labeled on the proteins using the isoxazolinium-mediated cysteine modification and a sequential azide-alkyne click reaction (Supplementary Fig. 9, 10).

Table 4 Enzymatic activities of native and modified BCArg

Sample	BCArg	BCArg-1	BCArg-2
Specific activity (U/mg)	109.77 ± 4.95	98.51 ± 13.95	99.64 ± 6.11
IC_{50} values for MDA-MB-231 (U/mL)	3.578 ± 1.278	4.719 ± 1.438	3.144 ± 1.232
IC_{50} values for NCI-H23 (U/mL)	3.921 ± 1.318	5.295 ± 1.262	5.146 ± 1.475

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 (λ_{max} = 365 nm) for 15 min, cysteine-modified peptide **3a** was converted to thioaldehyde product **10** in 41% conversion, while the

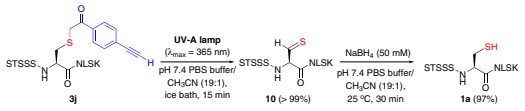


Fig. 5 Modification photolysis and sequential reduction of the modified peptide **3i**. Reduction regenerates the original cysteine residue

phenylacyl moiety was cleaved via a Norrish type II photolysis reaction (Supplementary Fig. 11). By introduction of an alkyne 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**. Conducting 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 $NaBH_4$ (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 isoxazoliniums 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 isoxazolinium reagents with a catalytic amount of silver salts were needed to give high-to-excellent conversions. Besides, easily accessible isoxazolinium 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 isoxazolinium 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 isoxazolinium reagents could be potentially employed as promising reagents for labeling bioactive proteins in the future.

We have also found that incorporation of an alkyne moiety on the phenylacyl thioether linkage would induce a rapid photolysis under irradiation of UV-A light ($\lambda_{max} = 365$ 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 photocleavable 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 isoxazolinium reagents 2a-u. For preparation of the isoxazolinium reagents **2a–t**, a mixture of propargylamines **I-a-t** (0.05 mmol, 1 equiv.) and *meta*-chloroperoxybenzoic acid (*m*-CPBA, 0.05 mmol, 1 equiv.) was conducted in a solution of CH_3CN (0.50 mL) and H_2O (0.40 mL) at 25 °C for 15 min, giving the formation of propargylamine *N*-oxides **II-a–t** in situ. After that, 0.10 mL of $AgNO_3$ solution (25 mM in H_2O) was added to the mixture and the reaction was conducted at 25 °C for 2 h to afford the isoxazolinium reagents **2a–t** (50 mM in CH_3CN/H_2O (1:1)). The reagents were further diluted to 5 mM in CH_3CN/H_2O (1:1) and stored at −20 °C for repeated usage. For preparation of isoxazolinium reagents **2u**, the mixture of propargylamine **I-u**, *m*-CPBA, and $AgNO_3$ was treated using the aforementioned conditions except for two equivalents of *m*-CPBA was used.

Modification of peptides using isoxazolinium reagents 2a–t. A mixture of 10 μL of peptides **1a–i** (1 mM in H_2O), 2 μL of isoxazolinium regents **2a–q** (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 4 μL of CH_3CN, 4 μL of H_2O, 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 isoxazolinium regent **2r**, the modification was conducted using the aforementioned conditions except that 3 μL of isoxazolinium regents **2r** (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 3.5 μL of CH_3CN, and 3.5 μL of H_2O were added. For modification of cysteine-containing peptide **1a** with fluorescein-derived isoxazolinium regent **2s**, the modification was conducted using the aforementioned conditions, except that 5 μL of isoxazolinium regents **2s** (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 2.5 μL of CH_3CN and 2.5 μL of H_2O were added.

Macrocyclization of peptides using isoxazolinium reagents 2u. A mixture of 10 μL of peptides **1j-k** (1 mM in H_2O), 2 μL of isoxazolinium regents **2u** (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 4 μL of TCEP (12.5 mM in H_2O), 4 μL of CH_3CN, 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 STSSSCNLSK 1a. A mixture of 20 μL of peptides **1a** (1 mM in H_2O), 4 μL of isoxazolinium regent **2a** (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 8 μL of CH_3CN, 8 μL of H_2O, 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, 10 μL of the resulting mixture was collected and mixed with 10 μL of L-cysteine (50 mM in H_2O) to quenched the modification. The resulting mixture was characterized by LC-MS and LC-MS/MS analysis to determine the conversion.

Modification of proteins using isoxazolinium reagents 2a and 2p. A mixture of 10 μL of proteins (**HSA**, **BSA**, insulin, RNaseA, or lysozyme) (1 mM in 50 mM pH 7.4 PBS buffer), 2 μL of isoxazolinium reagents **2a** or **2p** (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 4 μL of CH_3CN, 4 μL of H_2O, 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 isoxazolinium reagents 2a or 2p (5 mM in CH_3CN/H_2O containing 5 mol% $AgNO_3$), 25 μL of CH_3CN, 25 μL of H_2O, 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 −15 10 -K centrifugal device. After that, the filter was filled with 15% CH_3CN/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 RCF 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 buffer was used instead of 15% acetonitrile/50 mM pH 7.4 buffer at the last time. Modified proteins (0.1 mM) in 50 mM pH 7.4 buffer were collected.

Sequential modification of proteins via Huisgen azide-alkyne cycloaddition. A mixture of 50 μL of alkyne-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 TBTA (5 mM in DMSO), 5 μL of TCEP (5 mM in H_2O), 5 μL of $CuSO_4$ solution (5 mM in H_2O) 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 alkyne-functionalized proteins **BCArg-2**, 50 mM pH 7.4 Tris-HCl buffer was used instead of PBS buffer.

Procedure for photocleavage 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 AMP). For photocleavage 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/CH_3CN (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 ($\lambda_{max} = 365$ 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 (19:1)) 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 ($\lambda_{max} =$ 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.

Additional information

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