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# Electron-Deficient Alkynes as Cleavable Reagents for the Modification of Cysteine-Containing Peptides in Aqueous Medium

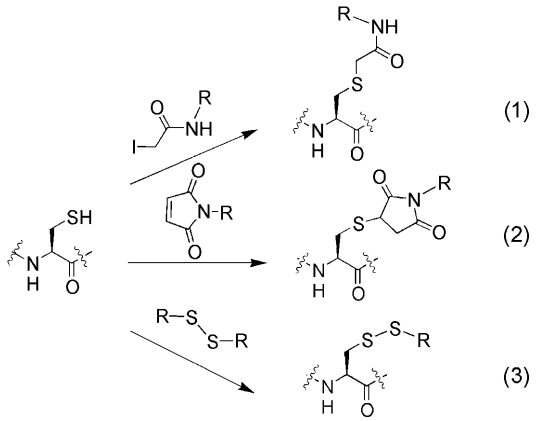
**Hoi-Yan Shiu, Tak-Chung Chan, Chi-Ming Ho, Yungen Liu, Man-Kin Wong,* and Chi-Ming Che*[a]**

**Abstract:** An efficient method has been developed for the chemoselective cysteine modification of unprotected peptides and proteins in aqueous media through the formation of a vinyl sulfide linkage by using electron-deficient alkynes, including alkynoic amides, esters and alkynones. The terminal alkynone-modified peptides could be converted back into the unmodified peptides (81% isolated yield) by adding thiols under mild conditions. The usefulness of this thiol-assisted cleavage of the vinyl sulfide linkage in peptides has been exemplified by the enrichment of a cysteine-containing peptide (71% recovery) from a mixture of cysteine-containing and non-cysteine-containing peptides.

**Keywords:** alkynes · chemoselectivity · cleavage reactions · cysteine · peptides

## Introduction

Site-selective modification of proteins is of growing importance because it leads to novel bioconjugates that are useful for biological studies of, for example, protein–protein interactions and live cell imaging.[1] Despite the advancements in chemical synthesis over the past few decades, there are only a handful of synthetic methods that can be applied to protein modification. Most of the chemical reactions used in organic synthesis are not compatible with aqueous media or the sensitive functional groups of proteins. Thus, there is a continued interest in the development of new methods for the selective modification of peptides and proteins under mild conditions.[2] Selective cysteine modification is a particularly useful bioconjugation reaction owing to the high reactivity of the cysteine sulfhydryl group and to the relatively sparse occurrence (1.7%) of cysteine units in peptides and proteins.[1] The reagents commonly used for cysteine modification can be divided into two categories. Alkylating reagents form the first category, including haloacyl compounds and maleimides, which modify cysteine units by nucleophilic substitution and conjugate addition reactions, respectively, as depicted by Equations (1) and (2) of Scheme 1. Both




Scheme 1. Methods for cysteine modification.

methods involve sulfur–carbon bond formation. However, cross-reactivity with other amino acids such as histidine, methionine, lysine and tyrosine has been reported, especially in media at high pH.[1] Thiol–disulfide exchange reagents are another class of reagents used for cysteine modification and they act through the reversible formation of a sulfur–sulfur bond [Scheme 1, Eq. (3)]. They can lead to specific modifi-

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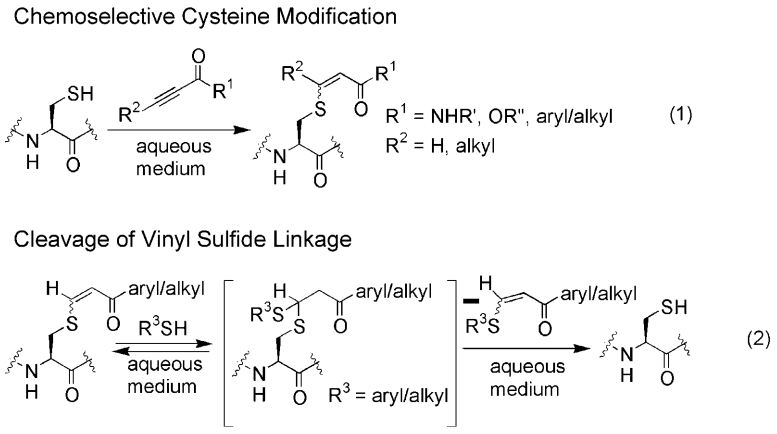
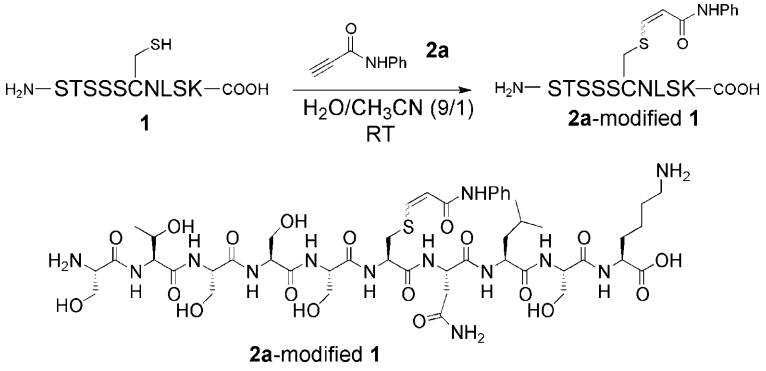
cation of biomolecules, but the inherent instability of the disulfide bond limits their applications in this endeavour. Kent and co-workers pioneered the selective coupling of N-terminal cysteine residues with C-terminal thioesters (native chemical ligation) to achieve chemical protein synthesis.[3] Recently, Crich and co-workers reported the use of allylic selenosulfides for chemical ligation of cysteine derivatives in protic media.[4] Despite these advances, the development of new reagents for the cysteine modification of peptides and proteins with chemoselectivity remains a challenge.

Conjugate addition of thiols to electron-deficient alkynes has been described in the literature, but most of the reported reactions were conducted in organic solvents.[5] Tsou and co-workers reported selective cysteine modifications by a series of reactions conducted in aqueous medium. The alkynoic amides could function as irreversible inhibitors of epidermal growth factor receptor kinase (EGFR) by forming a covalent linkage with the sulfhydryl group of the cysteine of the enzyme.[6] Arjona and co-workers reported a cleavage reaction for the deprotection of an electron-withdrawing tosylvinyl group from a thiol. The corresponding cleavage of the vinyl sulfide linkage was achieved by a base-induced thiolate exchange in organic solvents.[7]

Herein, we report the use of electron-deficient alkynes such as alkynoic amides, esters and alkynones for chemoselective cysteine modification of unprotected peptides through the formation of a vinyl sulfide linkage in an aqueous medium [Scheme 2, Eq. (1)]. Notably, with $R^1$ = aryl/alkyl and $R^2$ = H, the vinyl sulfide linkage could be cleaved to regenerate the unmodified peptides by treatment with thiols under mild conditions [Scheme 2, Eq. (2)]. The usefulness of this cleavage reaction has been demonstrated by the enrichment of a cysteine-containing peptide from a mixture of cysteine-containing and non-cysteine-containing peptides.




Scheme 2. Chemoselective cysteine modification and cleavage of the vinyl sulfide linkage.




Scheme 3. Modification of peptide **1** by alkyne **2a**.

## Results

**Cysteine-selective modification of peptide STSSSCNLSK (1) by electron-deficient alkynes**: Initially, the coupling reaction of an unprotected peptide STSSSCNLSK (**1**; 0.1 mM) with alkynoic amide **2a** (0.1 mM) in 100 μL of $H_2O/CH_3CN$ (9:1) at room temperature was examined (Scheme 3).[8] A 25% conversion of **1** to **2a**-modified **1** was observed after 500 min by HPLC analysis. Selective attachment of **2a** to the cysteine sulfhydryl group of **1** was confirmed by LC–MS/MS. Neither the N-terminal α-amino group nor the side-chains of serine and lysine were modified. Note that conjugate addition of the nucleophilic functional groups of serine, cysteine and lysine derivatives to alkynoic amides and esters and alkynones in organic solvents have been reported.[9] However, no selectivity of cysteine over serine or lysine derivatives in the reported coupling reactions was mentioned.

The conjugate addition of thiols to electron-deficient alkynes is generally facilitated in solutions at alkaline pH, for example, by the addition of imidazole as a base or by using alkaline Tris-HCl buffer (pH 7.0–9.0). Presumably this is due to the increase in concentration of thiolate ions in alkaline solution. As depicted in Figure 1, the reaction rate of the coupling reaction depicted in Scheme 3 increased with pH. Up to 80% conversion of **1** could be obtained when the reaction was conducted in a pH 8.0 buffer for 500 min.

The cysteine modification of peptide **1** also proceeded selectively with other electron-deficient alkynes, including alkynoic ester **3a** and alkynones **4a–d**, to give **3a**- and **4a–d**-modified **1**, respectively (Scheme 4).

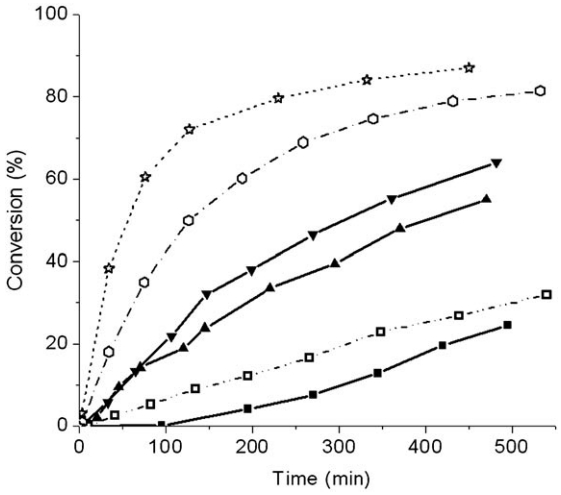



Figure 1. Time course of the modification of peptide **1** by alkyne **2a** under various conditions. ■: **2a** (0.1 mM), pH 6.3; ▲: **2a** (0.1 mM), imidazole (0.2 mM), pH 7.4; ▼: **2a** (0.1 mM), imidazole (1 mM), pH 7.7; □: **2a** (0.1 mM), pH 7.0 buffer; ○: **2a** (0.1 mM), pH 8.0 buffer; ☆: **2a** (0.1 mM), pH 9.0 buffer.

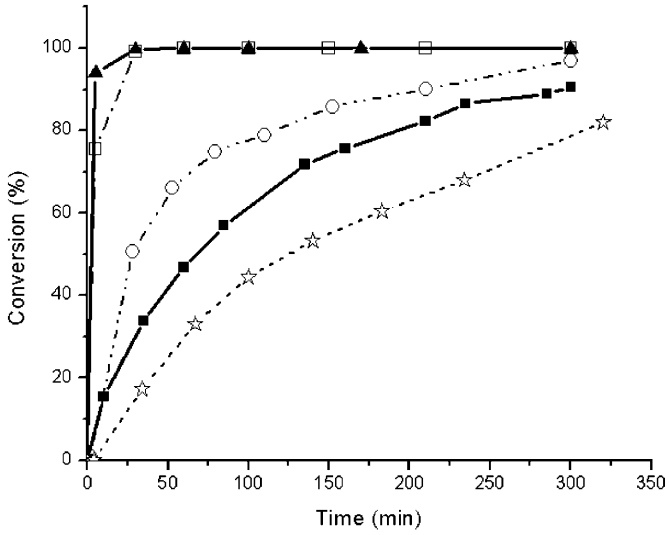
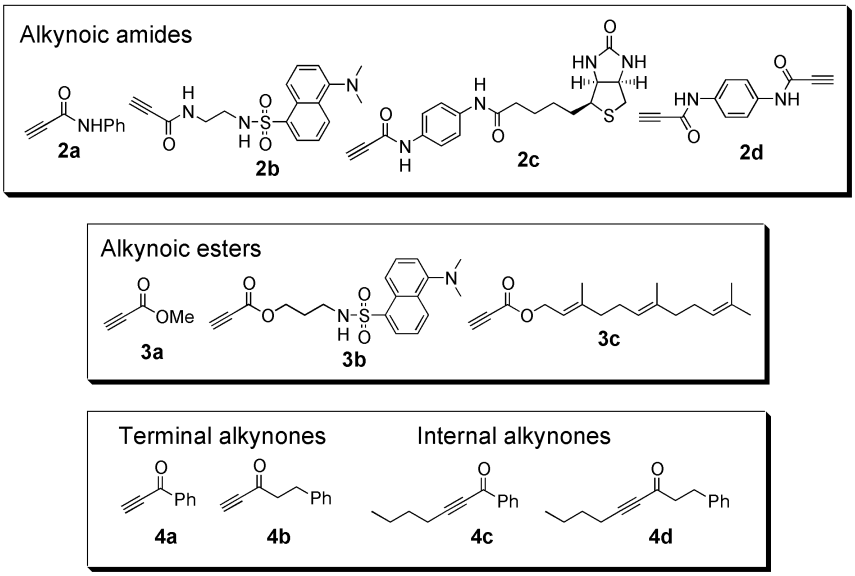



Figure 2. Time course of the modification of **1** with **3a** and **4a–d**. ■: **3a** (0.1 mM), pH 8.0 buffer; ▲: **4a** (0.1 mM), pH 8.0 buffer; □: **4b** (0.1 mM), pH 8.0 buffer; ○: **4c** (0.1 mM), pH 8.0 buffer; ☆: **4d** (0.1 mM), pH 8.0 buffer.





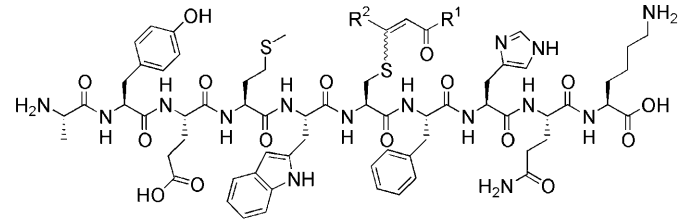
**2a**: $R^1$ = NHPh, $R^2$ = H
**3a**: $R^1$ = OMe, $R^2$ = H
**4a**: $R^1$ = Ph, $R^2$ = H
**4b**: $R^1$ = $CH_2CH_2Ph$, $R^2$ = H
**4c**: $R^1$ = Ph, $R^2$ = $n$-$C_4H_9$
**4d**: $R^1$ = $CH_2CH_2Ph$, $R^2$ = $n$-$C_4H_9$

Scheme 4. Structures of **2a**-, **3a**- and **4a–d**-modified **1**.

Figure 2 shows the reaction rate profile of the reactions between peptide **1** (0.1 mM) and alkynes **3a** and **4a–d** (0.1 mM). The reaction medium was Tris-HCl buffer (pH 8.0) with 10% (v/v) of $CH_3CN$ as co-solvent. A high initial reaction rate was observed in the modification of **1** by **4a** or **4b**. By using **4a**, over 90% conversion of **1** was obtained within 5 min. Modifications by both **4a** and **4b** could reach 100% conversion in 30 min. The reaction rates decrease in the following order: terminal alkynone ≫ internal alkynone ≈ alkynoic ester > alkynoic amide.

To examine the compatibility of electron-deficient alkynes with other nucleophilic amino acids, we conducted several coupling reactions with the unprotected peptide AYEMWCFHQK, which contains histidine, methionine, lysine and tyrosine, with different alkynes (Scheme 5).[10] The reactions of AYEMWCFHQK with **2a**, **3a**, **4a–d** and commonly used cysteine modification reagents (iodoacetamide and *N*-methylmaleimide) (1 equiv) in $H_2O$ and pH 8.0 buffer were attempted and exclusive cysteine modification was observed in all cases (Table 1). Note that only 1 equiv of **4a** or **4b** was required to achieve complete conversion in $H_2O$ (pH 6.3). The reactivities of **4a** and **4b** are comparable to that of *N*-methylmaleimide and yet significantly higher than that of iodoacetamide (<5% conversion). By using pH 8.0 buffer, a significant increase in the reaction rate was observed: 60% conversion for **2a** (entry 1) and complete conversion for all the other substrates (entries 2–8). When a large excess (50 equiv) of electrophile was used in pH 8.0



**2a**, **3a**, and **4a**-**d**-modified AYEMWCFHQK

**2a**: $R^1$ = NHPh, $R^2$ = H
**3a**: $R^1$ = OMe, $R^2$ = H
**4a**: $R^1$ = Ph, $R^2$ = H
**4b**: $R^1$ = $CH_2CH_2Ph$, $R^2$ = H
**4c**: $R^1$ = Ph, $R^2$ = $n$-$C_4H_9$
**4d**: $R^1$ = $CH_2CH_2Ph$, $R^2$ = $n$-$C_4H_9$

Scheme 5. Structures of **2a**-, **3a**-, **4a**–**d**-modified AYEMWCFHQK.

Table 1. Cysteine modification of AYEMWCFHQK.[a]

| Entry | Electrophile | Conversion [%][b] | | |
|---|---|---|---|---|
| | | $H_2O$ (1 equiv) | pH 8.0 (1 equiv) | pH 8.0 or 9.0 (50 equiv) |
| 1 | **2a** | <5 | 60 | 100 |
| 2 | **3a** | 30 | 100 | 100 |
| 3 | **4a** | 100 | 100 | 100[c] |
| 4 | **4b** | 100 | 100 | 100[c] |
| 5 | **4c** | <5 | 100 | 100 |
| 6 | **4d** | <5 | 100 | 100 |
| 7 | iodoacetamide | <5 | 100 | 100 |
| 8 | *N*-methylmaleimide | 100 | 100 | 100[c] |

[a] The reaction mixtures of AYEMWCFHQK and electrophiles in aqueous $CH_3CN$ solutions (10% $CH_3CN$) were maintained at 25 °C for 6 h. [b] The conversion [%] was determined by LC–MS/MS. [c] The amino acid residues of alanine (A) and cysteine (C) were modified.

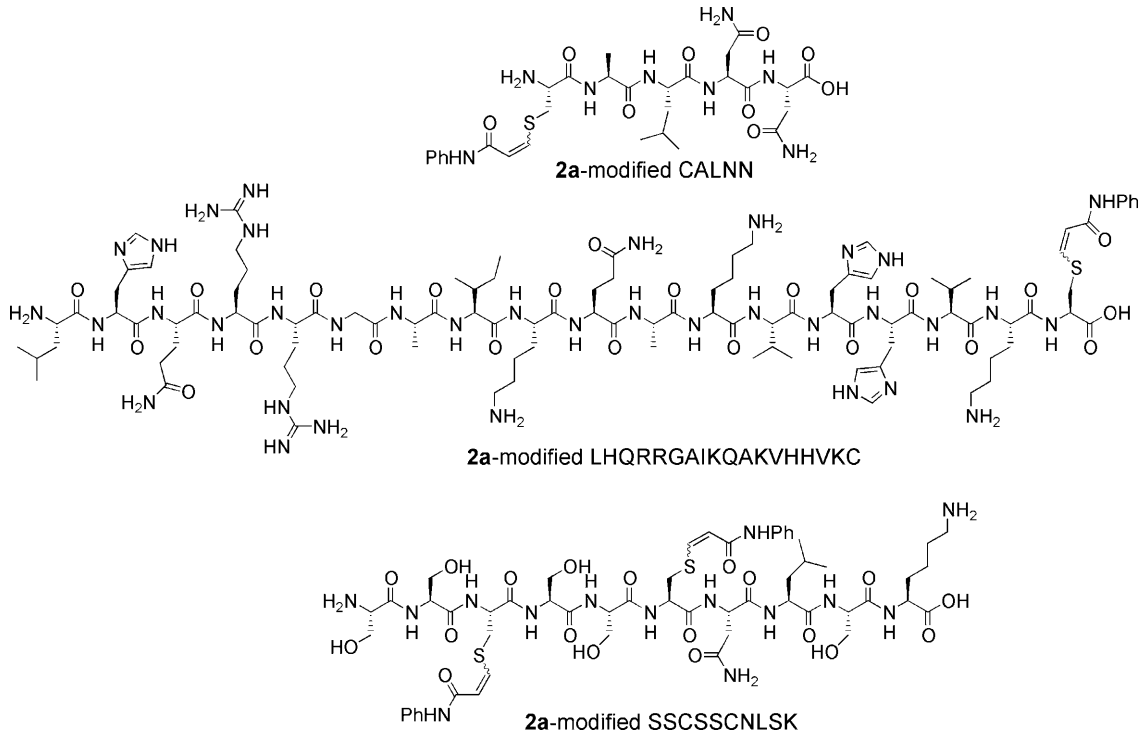
and 9.0 buffers, **2a**, **3a**, **4c**–**d** and iodoacetamide were able to give exclusive cysteine modification. In addition to cysteine modification, cross-reactivity with the N-terminal alanine residue was observed for **4a**, **4b** and *N*-methylmaleimide; however, the nucleophilic side-chains of histidine, methionine, lysine and tyrosine remained intact.

To demonstrate the practicality of this bioconjugation reaction, two one-pot peptide modifications were conducted; one by reacting 10 mg of AYEMWCFHQK with **2a** (1 equiv) in pH 8.0 buffer and the other with **4a** (1 equiv) in $H_2O$. Both reactions afforded the corresponding cysteine-modified peptides in 72% (7.9 mg) and 70% (7.6 mg) isolated yields, respectively.

Dansyl-, biotinyl- and farnesyl-linked alkynes **2b**,**c** and **3b**,**c** (5 equiv) individually attached to the cysteine unit of AYEMWCFHQK within 6 h in pH 8.0, which was confirmed by MALDI-TOF and LC–MS/MS analysis. Dialkyne-amide **2d** allowed coupling of two AYEMWCFHQK units through the cysteine side-chain.

By using **2a** in pH 8.0 buffer, cysteine modification of other unprotected peptides, including CALNN (N-terminal cysteine), LHQRRGAIKQAKVHHVKC (C-terminal cysteine) and SSCSSCNLSK (two cysteine units), was accomplished (Scheme 6).

**Modification of bovine serum albumin with fluorescent dansyl-linked alkyne 2b**: The ligation of the single, surface-exposed cysteine residue of bovine serum albumin (BSA) to fluorescent dansyl-linked alkyne **2b** was confirmed by MALDI-TOF MS analysis. The peaks at 66545 and



**2a**-modified CALNN

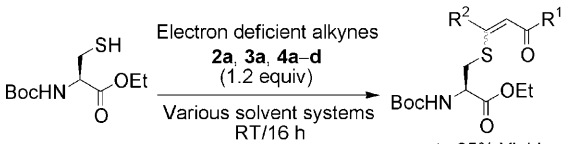
**2a**-modified LHQRRGAIKQAKVHHVKC

**2a**-modified SSCSSCNLSK

Scheme 6. Structures of **2a**-modified CALNN, LHQRRGAIKQAKVHHKC and SSCSSCNLSK.

66912 Da for native BSA and **2b**-modified BSA, respectively, revealed the addition of one molecule of **2b** per BSA. In addition, trypsin digestion of both the native BSA and the **2b**-modified BSA was conducted. The shift in the mass of the cysteine-containing peptide fragment Gly45-Lys65 (GLVLIAFSQYLQQCPF-DEHVK) from $m/z = 2435.2$ to 2780.3 after modification confirmed the incorporation of one molecule of **2b** (MW = 345) into the BSA peptide sequence. Notably, no modification was observed with lysozyme, which contains no free cysteine residue. It was found that **2b**-modified BSA gave a fluorescent signal under UV excitation in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) protein analysis. However, treatment of **2b** with lysozyme gave no fluorescent signal (Figure 3).

Table 2. *Z*/*E* ratios of **7**, **8** and **9a–d** obtained by the coupling of **6** with alkynes **2a**, **3a** and **4a–d**.[a]




**7**: $R^1$ = NHPh, $R^2$ = H
**8**: $R^1$ = OMe, $R^2$ = H
**9a**: $R^1$ = Ph, $R^2$ = H
**9b**: $R^1$ = $CH_2CH_2Ph$, $R^2$ = H
**9c**: $R^1$ = Ph, $R^2$ = *n*-$C_4H_9$
**9d**: $R^1$ = $CH_2CH_2Ph$, $R^2$ = *n*-$C_4H_9$

| Conditions | *Z*/*E* ratio of alkyne-modified **6**[b] | | | | | |
|---|---|---|---|---|---|---|
| | **7** | **8** | **9a** | **9b** | **9c** | **9d** |
| $H_2O/CH_3CN$ | 4:1[c] | 1:2 | 3:1 | 2:1 | 4:1 | 1:2 |
| $H_2O/CH_3CN$ + imidazole[d] | 8:1 | 6:1 | 5:1 | 3:1 | 3:1 | 1:1 |
| pH 7.0[e] | 16:1 | 5:1 | 5:1 | 3:1 | 2:1 | 1:1 |
| pH 8.0[e] | 18:1 | 8:1 | 5:1 | 2:1 | 2:1 | 2:1 |
| pH 9.0[e] | 22:1 | 5:1 | 5:1 | 1:1 | 3:1 | 2:1 |

[a] Reaction conditions: **6** (0.4 mmol), alkyne **2a**, **3a**, **4a–d** (0.48 mmol) in $H_2O$ (9 mL) and $CH_3CN$ (1 mL), $t = 16$ h, room temperature unless otherwise stated. [b] Determined by $^1H$ NMR analysis of the crude reaction mixture. [c] Incomplete conversion after 16 h reaction. [d] Imidazole (0.8 mmol) was added. [e] $H_2O$ was replaced by 9 mL of pH 7.0, 8.0 or 9.0 Tris-HCl buffer, respectively.

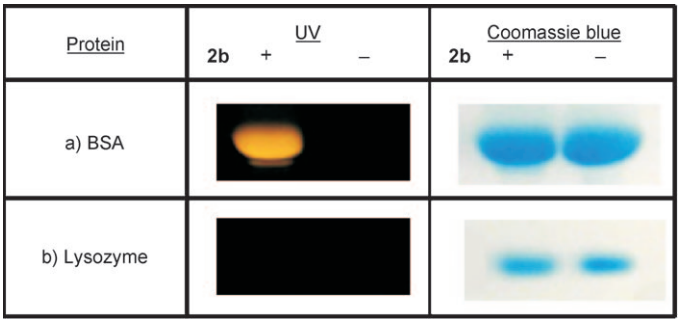



Figure 3. SDS-PAGE analysis of **2b**-modified BSA.

**Model studies of the stereochemistry of the vinyl sulfide linkages formed in peptide ligation**: As mentioned in the previous section, vinyl sulfide linkage was formed in the modification of peptides and proteins with electron-deficient alkynes. To model the stereoselectivity of the vinyl sulfide linkage formed in the peptide modification, coupling reactions of the protected cysteine **6** with alkynes **2a**, **3a** and **4a–d** were performed (Table 2). *Z* Stereoisomers were found to be the major coupling products for amide **7** (*Z*/*E* up to 22:1), ester **8** (*Z*/*E* up to 8:1) and ketones **9a–d** (*Z*/*E* up to 5:1). Our findings are consistent with previous work by Crisp and Millan who found that the ratio of *Z*/*E* stereoisomers formed in the conjugate addition of protected amino acids to electron-deficient alkynes was dependent on the solvent system used.[9a] In general, *Z* isomers are the predominant products formed from the *trans* addition of thiols to electron-deficient alkynes. Nevertheless, the electron-withdrawing group could lead to the isomerisation of the carbon–carbon double bond of the vinyl sulfide via intermediates such as allenol and ketene hemiacetal.[7b] In this connection, the weaker the electron-withdrawing ability of the activating group (alkynoic amides < alkynoic esters < alkynones), the less isomerisation of *Z* to *E* isomers would be observed. This trend is consistent with our findings: the *Z*/*E* ratios of the amide **7**, ester **8** and ketones **9a–d** are up to 22:1, 8:1 and 5:1, respectively.

**Cleavage reaction of electron-deficient alkyne-modified STSSSCNLSK by thiols**: A unique feature of the present method is that vinyl sulfide linkages are generated from the coupling of cysteine residues with alkynes. We envisioned that the unsaturated vinyl sulfide units may allow further synthetic manipulation. We examined the reactions of **2a**-, **3a**- and **4a–d**-modified **1** (Scheme 4) with thiols under different reaction conditions. As depicted in Figure 4, treatment of **4a**-modified **1** with excess PhSH (30 equiv) afforded the intermediate adduct **A**, which was then converted into **1**.

Similarly, the cleavage of **4a**-modified **1** to regenerate **1** was achieved in the presence of 1 equiv of imidazole or in alkaline buffer solutions (pH 7.0–9.0; Figure 5). The highest conversion (over 80%) was achieved by using pH 8.0 buffer and over 70% regeneration could be obtained in 25 min.

In addition, **4b**-modified **1** could be converted into **1** under the same reaction conditions. In contrast, **2a**-, **3a**- and **4c**,**d**-modified **1** and **2b**-modified BSA were stable in the presence of PhSH.

Of the nucleophiles examined, including $HOCH_2CH_2SH$, L-cysteine, PhOH, EtOH, PhSeH, $C_6H_{13}NH_2$ and two commonly used reducing agents, DTT (dithiothreitol) and TCEP (tris(2-carboxyethyl)phosphine), only $HOCH_2CH_2SH$, L-cysteine and DTT were able to regenerate the unmodified peptide.

The reactivities of the three thiols, PhSH, L-cysteine and $HOCH_2CH_2SH$, towards the cleavage of **4a**-modified **1** were examined and compared (Figure 6). The three thiols exhibited similar reactivity in the cleavage reaction (up to 45% re-

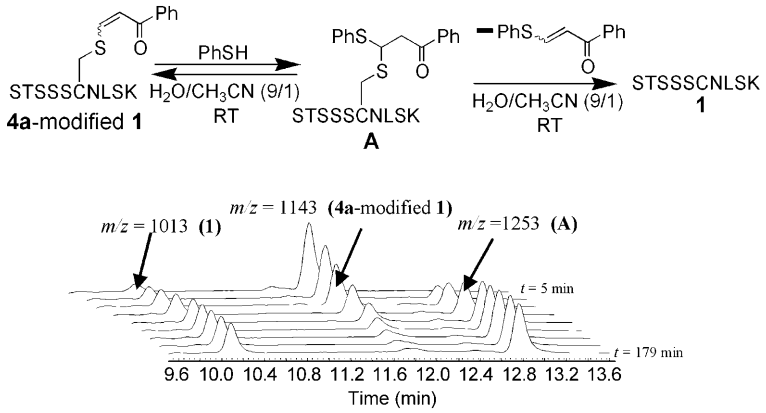



Figure 4. Cleavage of **4a**-modified **1** by PhSH.

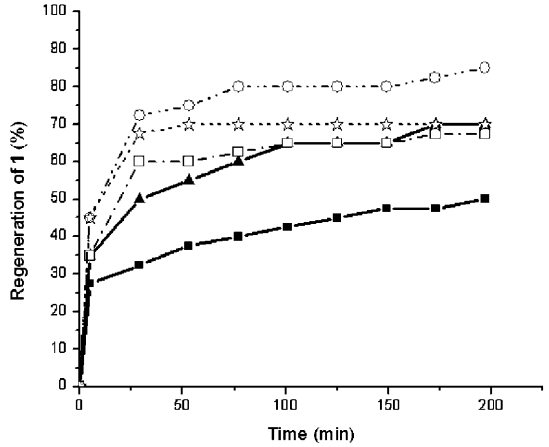



Figure 5. Cleavage of **4a**-modified **1** by PhSH under various conditions. ■: PhSH (3 mM), pH 6.3; ▲: PhSH (3 mM), imidazole (0.1 mM), pH 7.2; □: PhSH (3 mM), pH 7.0 buffer; ○: PhSH (3 mM), pH 8.0 buffer; ☆: PhSH (3 mM), pH 9.0 buffer.

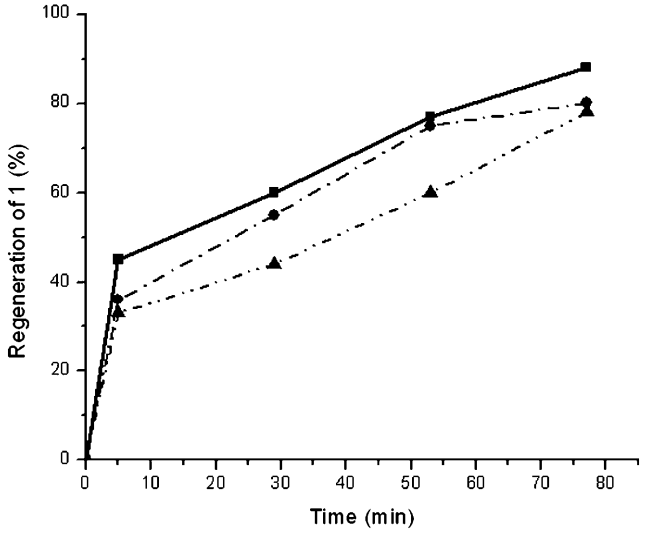



Figure 6. Cleavage of **4a**-modified **1** by three different thiols at pH 8.0. ■: PhSH (3 mM); ●: L-cysteine (3 mM), ▲: $HOCH_2CH_2SH$ (3 mM).

generation of **1** in 5 min and up to 85% regeneration of **1** in 75 min). PhSH was found to have a slightly higher reactivity than L-cysteine and $HOCH_2CH_2SH$.

Treatment of 10 mg of **4a**-modified **1** with 30 equiv of L-cysteine afforded the unmodified peptide in 81% isolated yield. The regeneration of the starting peptide from **4a**- and **4b**-modified AYEMWCFHQK was also accomplished by the addition of PhSH, $HOCH_2CH_2SH$ or L-cysteine.

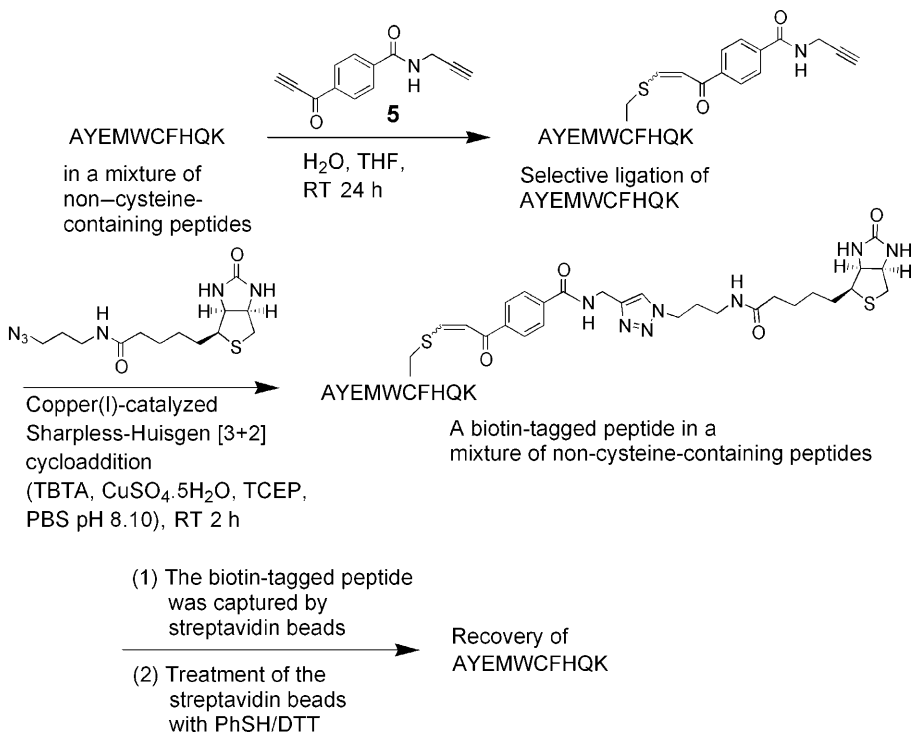
**Peptide enrichment using a cleavable biotin-linked alkyne**: In proteomics applications, the enrichment of specific peptide targets from complex biological mixtures is important for reducing the complexity of peptide samples and for enhancing specific subsets of low-abundance proteins.[11] Cysteine is a widely used amino acid residue for the attachment of specific affinity tags for sample enrichment. This is generally achieved by covalent ligation of cysteine-containing peptides/proteins by biotin-linked tags. The biotin-tagged samples can be enriched by binding to immobilised streptavidin. However, harsh conditions, such as incubation of the biotin–streptavidin complex at 60–90 °C under denaturing conditions, are required to release the biotin-tagged samples.[12] On the other hand, on-bead digestion may lead to contamination by streptavidin, natively biotinylated proteins and/or non-specific resin-bound proteins.

In this work we found that exclusive ligation of alkyne **5** to AYEMWCFHQK occurred in the presence of a mixture of two non-cysteine-containing peptides, RPKPQQF and ASHLQLAR (Scheme 7). After coupling of the peptide mixture with a biotin azide by a copper(I)-catalysed Sharpless–Huisgen [3+2] cycloaddition reaction,[13] the biotin-tagged AYEMWCFHQK was subsequently captured by streptavidin beads. Treatment of the beads with an aqueous solution of PhSH/DTT at pH 8 at 37 °C for 2 h regenerated AYEMWCFHQK, as confirmed by LC–MS/MS analysis. A 71% recovery of the peptide AYEMWCFHQK was achieved.

## Discussion

**Electron-deficient alkynes as a new class of cleavable reagents for chemoselective cysteine modification**: Conjugate addition of thiols to electron-deficient alkynes under alkaline conditions has been known for decades. However, the reported reactions involved simple thiols and were conducted in organic solvents.[5] Apart from thiols, amines and alcohols can also react with alkynes. In this work electron-defi-




Scheme 7. Peptide enrichment experiment.

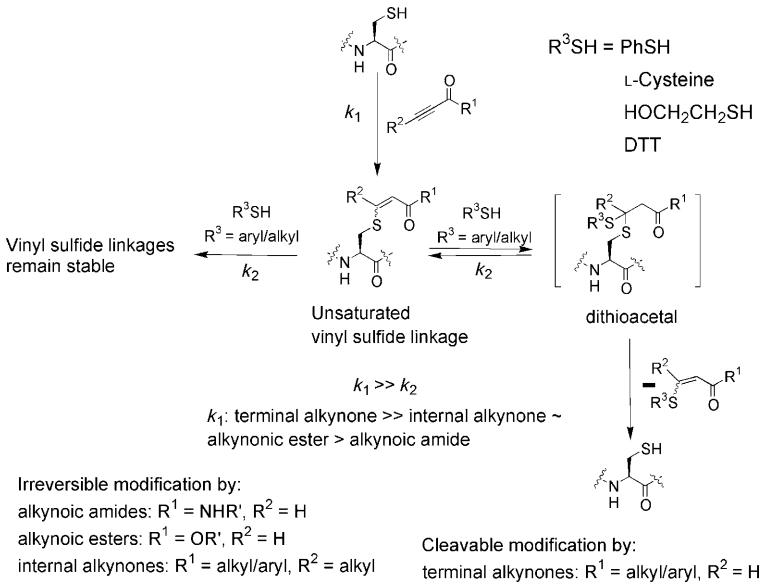
cient alkynes have been found to selectively modify unprotected cysteine-containing peptides and proteins in aqueous media with other amino acid groups such as histidine, methionine, lysine and tyrosine remaining intact.

By using the present method, an unsaturated vinyl sulfide linkage is generated from the coupling of a cysteine residue with electron-deficient alkynes. We found that the unsaturated vinyl sulfide units could be cleaved by an addition/elimination mechanism by treatment with thiols. In contrast, the currently used alkylation reagents (haloacyl compounds and maleimides) modify cysteine by the formation of non-cleavable sulfur–carbon bonds.

By a judicious choice of alkyne substituents ($R^1$ and $R^2$), we were able to tune the reactivity of the alkynones towards the thiol-assisted cleavage of vinyl sulfide linkages (Scheme 8). In this work treatment of terminal alkynone-modified peptides with thiols led to the recovery of unmodified peptides under mild reaction conditions. In contrast, alkynoic amide-, alkynoic ester- and internal alkynone-modified peptides were stable in the presence of thiols leading to irreversible modification.

As depicted in Scheme 8, the key step in the cleavage reaction is the addition of the thiol to the vinyl sulfide linkage to give a dithioacetal, which subsequently releases the peptide by elimination. In general, the addition of thiol to vinyl sulfide ($k_2$) has a lower reaction constant than that for the coupling of the cysteine residue with the electron-deficient alkyne ($k_1$).[14] In this work the rates of cysteine modification by different alkynes are in the following order: terminal alkynone $\gg$ internal alkynone $\approx$ alkynoic ester $>$ alkynoic amide (Figure 2 and Scheme 5). Thus the formation of dithioacetal from the second conjugate addition of thiols to the less reactive alkynoic amide-, alkynoic ester- and internal alkynone-modified peptides would be less favourable, which accounts for their stability in the presence of excess thiols.

As mentioned previously, the conjugate addition of thiols to electron-deficient alkynes is generally assisted by the addition of imidazole as a base or by using alkaline Tris-HCl buffer (pH 7.0–9.0). Similarly, the cleavage of alkynone-modified peptides by thiols could also be facilitated in alkaline solution as the cleavage reaction involves a second addi-




Scheme 8. Different reactivities in the thiol-assisted cleavage of vinyl sulfide linkages.

tion of thiolate to the unsaturated vinyl sulfide linkage. Both the cysteine modification and cleavage reaction could be accelerated by an increase in thiolate ion concentration in alkaline solution.

In this work, alkynoic amides, including **2a–d**, were demonstrated to be efficient reagents with excellent chemoselectivity for the modification of cysteine-containing peptides/proteins. As depicted in Table 1, exclusive cysteine modification could be achieved even with a large excess (50 equiv) of alkynoic amides at pH 9.0. In contrast, cross-reactivity with the N-terminal α-amino group of alanine was observed for the commonly used *N*-methylmaleimide under the same reaction conditions. As depicted in Table 2, high stereoselectivity (*Z*/*E* up to 22:1) for vinyl sulfide formation could be obtained in alkaline solution. This high stereoselectivity is important for minimising the formation of heterogeneous mixtures of small molecule–biomolecule conjugates. Our studies indicated that alkynoic amides tolerated a wide range of reaction conditions: 1 or 50 equiv of reagent used, $H_2O$, imidazole or pH 7.0–9.0 buffer, and room temperature to 37 °C. The practicality of alkynoic amides in bioconjugation has been exemplified by the following one-pot peptide modification: the reaction of 10 mg of AYEMWCFHQK with **2a** (1 equiv) in pH 8.0 buffer afforded the corresponding cysteine-modified peptide in 72% (7.9 mg) isolated yield. Finally, the alkynoic amide modified peptides remained stable upon treatment with thiols and reducing agents, including DTT and TCEP, under various conditions.

Terminal alkynones, including **4a** and **4b**, exhibited the highest reactivity in cysteine modification. As shown in Figure 2, when **4a** (1 equiv) in pH 8.0 buffer was used, over 90% conversion of **1** was obtained within 5 min and 100% substrate conversion was achieved in 30 min. One-pot peptide modification by the reaction of 10 mg of AYEMWCFHQK with **4a** (1 equiv) in $H_2O$ gave the cysteine-modified peptide in 70% (7.6 mg) isolated yield. The high reactivity of terminal alkynones allows high-throughput cysteine modification and obviates the need of using excess labelling reagents. Notably, we found that terminal alkynone-modified peptides could be converted back into unmodified peptides by thiol-assisted cleavage under mild reaction conditions. Note that treatment of 10 mg of **4a**-modified **1** with 30 equiv of L-cysteine afforded the unmodified peptide in 81% isolated yield. In this preparative-scale cleavage reaction, odourless L-cysteine could be used instead of the strongly odorous PhSH. Indeed, $HOCH_2CH_2SH$, a commonly used reagent in biological experiments, could also be employed.

Most of the alkynes used in our experiments are not commercially available, but they can be prepared by relatively simple procedures, as depicted in the Supporting Information. Alkynoic amides **2** and alkynoic esters **3** could be prepared in good yields by coupling propiolic acid or propynoyl chloride with amines and alcohols, respectively. Alkynones **4** could be synthesised by nucleophilic addition of lithiated alkynes/Grignard reagents to aldehydes followed by alcohol oxidation. The functionalised alkynes such as dansyl-, biotinyl- and farnesyl-linked alkynes **2b**,**c** and **3b**,**c** were prepared by simple organic reactions. By using click chemistry, the **5**-modified peptides could be further functionalised by coupling with organic azides, allowing a convenient access to a wide variety of structurally diverse bioconjugates. Given their excellent chemoselectivity in cysteine modification and ease of synthesis, we envision that electron-deficient alkynes could be developed into a class of reliable and practical reagents for cysteine modification.

**The use of a cleavable biotin-linked alkyne in peptide enrichment experiments**: In this work the use of electron-deficient alkynes allowed modification of cysteine-containing peptides and proteins in a selective manner. We have therefore developed a cleavable biotin-linked alkyne for specific enrichment of cysteine-containing peptide samples with high recovery (71%) under mild conditions (Scheme 7). Cleavage of the vinyl sulfide linkage is thiol-specific and yet the vinyl sulfide linkage remains stable upon treatment with phosphine-based reducing reagents such as TCEP. This orthogonal reactivity towards phosphine-based reducing reagents allows sample preparation for proteomics applications using phosphine-based reducing reagents. In particular, this would allow subsequent modification of the alkyne-modified peptides by copper(I)-catalysed Sharpless–Huisgen [3+2] cycloaddition in which TCEP is needed for in situ generation of copper(I) ions. The enrichment procedure described in this work is compatible with the conventional biotin–streptavidin affinity technique.[12b] After cleavage of the vinyl sulfide linkage, the free cysteine sulfhydryl group is recovered in the enriched peptides. Thus, the use of these cleavable biotin-linked alkynes could lead to the recovery of the native peptides/proteins. This would simplify subsequent mass spectrometry analysis of the enriched samples by eliminating undesirable effect(s) associated with tagging groups such as reduced sensitivity and tag fragmentation.

An important factor governing the utility of these cleavable reagents in peptide enrichment experiments is the kinetics of reversibility of the biotinylated peptides. In this work no scrambled product was found when biotinylated peptide AYEMWCFHQK was treated with a mixture of peptides containing free cysteine residues. Furthermore, the biotinylated peptide (60 μM) as well as the **4a**- and **5**-modified peptides (10 μM) in pH 8.0 Tris-HCl buffer/$CH_3CN$ (9:1) remained stable after storage at 25 °C for 30 days. These experiments revealed that cleavable electron-deficient alkynones should have potential in proteomics studies of complex biological samples.

Biotin disulfide *N*-hydroxysuccinimide ester (Biotin-S-S-NHS) is a commercially available biotin–disulfide linker used for the recovery of protein–DNA complexes after avidin affinity column purification.[15] The NHS moiety allows ligation of the probe to the amino groups of the target proteins, whereas the biotin group is used for specific binding onto the streptavidin beads. Cleavage of the disulfide bond could be achieved by treatment with thiol-based

reducing agents. However, the NHS group is amine-reactive such that lysine and α-amino groups can react with the linker leading to non-specific modification. There is no selectivity in the cleavage of the disulfide linker by thiol- and phosphine-based reducing agents. Proteins obtained after cleavage of the disulfide linker are attached with an amine-modified residue that precludes the recovery of native proteins and causes complications in subsequent mass spectrometric analyses.

## Conclusion

We have developed an efficient and versatile method for the selective cysteine modification of unprotected peptides and proteins in aqueous media using electron-deficient alkynes through the formation of a vinyl sulfide linkage. Notably, peptides modified by terminal alkynones could be converted back into the unmodified peptides by treatment with thiols under mild reaction conditions. The usefulness of this thiol-assisted cleavage of the vinyl sulfide linkage has been demonstrated by the selective enrichment of a cysteine-containing peptide from a mixture of peptides.

## Experimental Section

**General**: Chemicals purchased from commercial sources were used without further purification. Double-distilled water ($ddH_2O$) used as reaction solvent and in peptide modifications was deionised by using a NANOpure purification system (Barnstead, USA). Flash column chromatography was performed by using silica gel 60 (230–400 mesh ASTM) with ethyl acetate/*n*-hexane or methanol/dichloromethane as eluent. HPLC analysis was conducted by using a Waters 2690 Separations Module equipped with a Waters 996 Photodiode Array Detector and a XTerra RP18 5 μm column. $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker DPX-300 or DPX-400 spectrometer. Chemical shifts (ppm) are referenced to TMS. Mass spectra were measured by using a Finnigan MAT 95 or LCQ mass spectrometer.

**Mass spectrometric analysis of peptides**: All peptide samples for MALDI-TOF MS analysis were co-crystallised by using a α-cyano-4-hydroxycinnamic acid (Aldrich) solution (10 mg mL$^{-1}$ $CH_3CN/ddH_2O$ (1:1) with 0.1% TFA). MALDI-TOF mass spectra of peptides were recorded in reflectron mode by using a Voyager-DE STR system (ABI, USA) equipped with a $N_2$ laser (337 nm). LC–MS/MS analyses were performed by using a hybrid Q-TOF mass spectrometer (QSTAR-XL system, ABI, USA) equipped with an ionspray source and an Agilent 1100 series cap-LC pump. Chromatography of the reaction mixture was performed by using an Agilent ZORBAX 300SB-C18 (0.3 mm × 150 mm) reversed-phase column with a $CH_3CN/ddH_2O$ gradient mobile phase containing 0.1% formic acid (flow rate: 5 μL min$^{-1}$).

**Solid-phase synthesis of peptides**: Peptides were synthesised according to the standard solid-phase Fmoc-peptide synthesis procedure using the Wang resin as solid support. The peptides were purified by preparative reversed-phase HPLC using a $C_{18}$ column with $CH_3CN/H_2O$/TFA as the solvent system. The amino acid sequences of the peptides were confirmed by tandem mass spectrometric (MS/MS) analysis.

The peptides STSSSCNLSK, AYEMWCFHQK, CALNN and SSCSSCNLSK were synthesised, whereas the peptides LHQRRGAIKQAKVHHVKC, ASHLGLAR and RPKPQQF were obtained from commercial sources.

**Procedure for the modification of peptide 1 by electron-deficient alkyne 2a**: In a 1 mL vial equipped with a 500 μL insert, peptide **1** (40 μL, 1 μmol mL$^{-1}$ in $H_2O$), electron-deficient alkyne **2a** (4 μL, 10 μmol mL$^{-1}$ in $CH_3CN$), $H_2O$ (320 μL) and $CH_3CN$ (36 μL, final volume 400 μL, $H_2O/CH_3CN = 9:1$) were mixed at room temperature. The reaction mixture was then monitored by reversed-phase HPLC. Every 30 min, a sample of the reaction mixture (30 μL) was injected into the HPLC apparatus. The peptides were eluted at a flow rate of 0.5 mL min$^{-1}$ with the solvent gradient shown in Figure S1 of the Supporting Information and the column effluent was monitored at 210 nm. The peptide sequences were determined by LC–MS/MS.

The above coupling reaction between **1** and **2a** was repeated under various conditions and was also performed with **1** and **3a** as well as **4a–d** at pH 8.0 (see the Supporting Information).

**Procedure for the coupling reaction of protected cysteine 6 with the electron-deficient alkyne 2a**: Imidazole (55 mg, 0.8 mmol) was added to a round-bottom flask containing **6** (100 mg, 0.4 mmol) and alkyne **2a** (70 mg, 0.48 mmol) in a solution of $H_2O$ (9 mL) and $CH_3CN$ (1 mL). After stirring at room temperature for 16 h, the reaction mixture was extracted with $CH_2Cl_2$ (3 × 20 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (30–40% EtOAc in *n*-hexane) to provide (*Z*)-/(*E*)-**7** (142 mg, 90% isolated yield based on 100% conversion) as a solid.

The above reaction conditions were used for the coupling of **6** with **3a** and **4a–d**.

**General procedure for cysteine modification of AYEMWCFHQK with 2a, 3a, 4a–d, iodoacetamide and *N*-methylmaleimide (1 or 50 equiv) in $H_2O$ or pH 8.0 Tris-HCl buffer**: AYEMWCFHQK (10 μL, 1 μmol mL$^{-1}$ in $H_2O$), electrophile (1 μL, 1 equiv from a stock solution: 10 μmol mL$^{-1}$ in $CH_3CN$), $CH_3CN$ (9 μL) and $H_2O$ or pH 8.0 Tris-HCl buffer (80 μL) were mixed in a 1.0 mL Eppendorf tube. When 50 equiv of alkyne was needed, 5 μL of the electrophile (from a stock solution: 100 μmol mL$^{-1}$ in $CH_3CN$) was used. The reaction mixture was kept at 25 °C for 6 h. The cysteine-modified peptides were characterised by LC–MS/MS.

**General procedure for cysteine modification of N- and C-terminal cysteine-containing peptides (CALNN and LHQRRGAIKQAKVHHVKC) with 2a**: The peptide (10 μL, 1 μmol mL$^{-1}$ in $H_2O$), **2a** (1 μL, 10 μmol mL$^{-1}$ in $CH_3CN$), $CH_3CN$ (9 μL) and pH 8.0 Tris-HCl buffer (80 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 6 h. The cysteine-modified peptides were characterised by LC–MS/MS.

**Procedure for cysteine modification of SSCSSCNLSK with 2a**: The peptide (10 μL, 1 μmol mL$^{-1}$ in $H_2O$), **2a** (10 μL, 10 μmol mL$^{-1}$ in $CH_3CN$) and pH 8.0 Tris-HCl buffer (80 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 6 h. The cysteine-modified peptide was characterised by LC–MS/MS.

**General procedure for cysteine modification of AYEMWCFHQK with 2b–d and 3b,c**: The peptide AYEMWCFHQK (10 μL, 1 μmol mL$^{-1}$ in $H_2O$), alkyne (5 μL, 10 μmol mL$^{-1}$ in $CH_3CN$), $CH_3CN$ (5 μL) and pH 8.0 Tris-HCl buffer (80 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 6 h. The cysteine-modified AYEMWCFHQK was characterised by LC–MS/MS.

**Procedures for the large-scale synthesis of 2a- and 4a-modified AYEMWCFHQK**: A solution of the peptide AYEMWCFHQK (10 mg) and **2a** (2.3 mg, 1.5 equiv) in pH 8.0 Tris-HCl buffer/$CH_3CN$ (100 mL, 9:1) was prepared and placed in two centrifugal tubes (size 50 mL). The reaction mixtures were kept at 25 °C overnight. The aqueous phases were freeze-dried and the residues purified by preparative reversed-phase HPLC using a $C_{18}$ column and $CH_3CN/H_2O$/TFA as the solvent system. The **2a**-modified AYEMWCFHQK was isolated in 72% yield (7.9 mg). The sequence of **2a**-modified AYEMWCFHQK was confirmed by MALDI-TOF and LC–MS/MS analyses.

For the synthesis of **4a**-modified AYEMWCFHQK, a solution of AYEMWCFHQK (10 mg) and **4a** (2 mg, 1.5 equiv) in $H_2O/CH_3CN$ (100 mL, 9:1) was prepared. Following the procedure described above, **4a**-modified AYEMWCFHQK was isolated in 70% yield (7.6 mg).

**General procedure for modification of bovine serum albumin (BSA) and lysozyme with 2b**: In the case of BSA, a solution of BSA (10 μL, 1 μmol mL$^{-1}$ in $H_2O$), **2b** (10 μL, 10 μmol mL$^{-1}$ in $CH_3CN$) and pH 8.0 Tris-HCl buffer (80 μL) were mixed in a 1.0 mL Eppendorf tube. In the case of lysozyme, a solution of lysozyme (10 μL, 1 μmol mL$^{-1}$ in $H_2O$), **2b** (10 μL, 10 μmol mL$^{-1}$ in $CH_3CN$) and pH 8.0 Tris-HCl buffer (80 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 16 h. The cysteine-modified BSA was subjected to SDS-PAGE analysis and trypsin digestion for MALDI-TOF analysis.

**Procedure for SDS-PAGE analysis**: The **2b**-modified protein mixture (10 μL) was mixed with 2X loading buffer (90 μL) in a 0.5 mL Eppendorf tube and then boiled for 5 min. Samples were analysed by SDS-PAGE by loading a sample of the boiled solution (10 μL) in each lane of a 12.5% SDS-PAGE gel and running in a SE 250 Mini-Vertical Unit (Amersham, USA) at 100 V at room temperature until the front of the dye reached the bottom of the gel. After SDS-PAGE separation, the **2b**-modified protein mixture was visualised with an Alphamager 2200 Multilmage Light Cabinet and finally stained with Coomassie blue.

**Procedure for trypsin digestion**: For the trypsin digestion of BSA, a ratio of 1:50 (w/w) of trypsin to BSA was used. The **2b**-modified BSA mixture (10 μL) was mixed with an ammonium bicarbonate solution (100 μL, 100 mM) in a 0.5 mL Eppendorf tube. The trypsin solution (1 μL, 1 mg mL$^{-1}$ in 1 mM HCl) was added to the Eppendorf tube. The reaction mixture was incubated at 37 °C for 2 h and the trypsin-digested mixture was then ready for MALDI-TOF analysis.

**Procedure for cleavage of 4a-modified 1 with PhSH to regenerate unmodified 1**

*Preparation of **4a**-modified **1***: Peptide **1** (40 μL, 1 μmol mL$^{-1}$ in $H_2O$), **4a** (4 μL, 10 μmol mL$^{-1}$ in $CH_3CN$), $CH_3CN$ (4 μL) and $H_2O$ (32 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 24 h. HPLC analysis confirmed that all of **1** had been consumed. A new peak was found at 11.7 min with $m/z = 1143$, which was assigned to **4a**-modified **1**. The **4a**-modified **1** was characterised by MALDI-TOF MS analysis.

The above procedure was repeated for the preparation of **4b**-modified **1** and for the modification of AYEMWCFHQK by **4a**.

*Cleavage reaction of **4a**-modified **1** by PhSH*: The **4a**-modified **1** (80 μL), PhSH (12 μL, 100 μmol mL$^{-1}$ in $CH_3CN$), $CH_3CN$ (28 μL) and $H_2O$ (280 μL) were mixed in a 1.0 mL Eppendorf tube at room temperature. The reaction mixture was then monitored by reversed-phase HPLC. Every 24 min, a sample of the reaction mixture (30 μL) was injected in the HPLC apparatus. The peptide was eluted at a flow rate of 0.5 mL min$^{-1}$ with the solvent gradient shown in Figure S1 of the Supporting Information (the column effluent was monitored at 210 nm) and characterised by MALDI-TOF MS analysis.

The above procedure was repeated for the cleavage of **4b**-modified **1** and **4a**-modified AYEMWCFHQK.

**General procedure for the cleavage reactions of 4a-modified 1 by nucleophiles to regenerate unmodified 1 in pH 8.0 Tris-HCl buffer**: The **4a**-modified **1** (20 μL), the water-soluble nucleophile stock solution (30 μL of $HOCH_2CH_2SH$ or L-cysteine), $CH_3CN$ (10 μL) and pH 8.0 Tris-HCl buffer (40 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 8 h. The reaction mixture was analysed by MALDI-TOF MS.

The above procedure was used for the cleavage of **4a**-modified AYEMWCFHQK by $HOCH_2CH_2SH$ or L-cysteine.

The **4a**-modified **1** (20 μL), the water-insoluble nucleophile stock solution (3 μL of PhOH, ethanol, PhSeH or hexylamine), $CH_3CN$ (7 μL) and pH 8.0 Tris-HCl buffer (70 μL) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 8 h. The reaction mixture was analysed by MALDI-TOF MS.

**Procedure for the large-scale synthesis of 4a-modified STSSSCNLSK and recovery of unmodified STSSSCNLSK**: A solution of peptide **1** (20 mg) and **4a** (2.6 mg, 1 equiv) in $H_2O/CH_3CN$ (200 mL, 9:1) was prepared and placed in four centrifugal tubes (size 50 mL). The reaction mixtures were kept at 25 °C overnight. The aqueous phases were freeze-dried and purified by preparative reversed-phase HPLC using a $C_{18}$ column and $CH_3CN/H_2O$/TFA as the solvent system. The **4a**-modified **1** was isolated in 55% yield (12.4 mg). The **4a**-modified **1** was confirmed by MALDI-TOF MS analysis.

A solution of **4a**-modified **1** (10 mg) and L-cysteine (32 mg, 30 equiv) in pH 8.0 Tris-HCl buffer/$CH_3CN$ (100 mL, 9:1) was prepared and placed in two centrifugal tubes (size 50 mL). The reaction mixtures were kept at 25 °C for 4 h. The aqueous phases were freeze-dried and purified by preparative reversed-phase HPLC using a $C_{18}$ column and $CH_3CN/H_2O$/TFA as the solvent system. The unmodified **1** was isolated in 81% yield (7.2 mg) and was confirmed by MALDI-TOF MS analysis.

**Procedure for peptide enrichment using a cleavable biotin-linked alkyne**: Alkyne **5** (6 μL, 10 μmol mL$^{-1}$ in THF), a mixture of peptides, RPKPQQF (60 μL, 1 μmol mL$^{-1}$ in $H_2O$), ASHLQLAR (60 μL, 1 μmol mL$^{-1}$ in $H_2O$) and AYEMWCFHQK (60 μL, 1 μmol mL$^{-1}$ in $H_2O$), and THF (14 μL, final volume of 200 μL with $H_2O$/THF = 9:1) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 24 h. The sequence of **5**-modified AYEMWCFHQK and the other two unmodified peptides were characterised by LC–MS/MS analysis.

The above **5**-modified peptide mixture (20 μL), phosphate-buffered saline (PBS; 58 μL, pH 8.10), biotin-$N_3$ (1 μL, 10 μmol mL$^{-1}$ in DMSO), tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 10 μL, 10 μmol mL$^{-1}$ in $H_2O$), tris(benzyltriazolylmethyl)amine (TBTA; 1 μL, 10 μmol mL$^{-1}$ in DMSO/*tert*-butyl alcohol = 1:4) and $CuSO_4 \cdot 5H_2O$ (10 μL, 10 μmol mL$^{-1}$ in $H_2O$) were mixed in a 1.0 mL Eppendorf tube. The reaction mixture was kept at 25 °C for 1 h. The sequence of the biotin-tagged AYEMWCFHQK and the other two unmodified peptides were characterised by LC–MS/MS and MALDI-TOF MS/MS analyses.

A solution of Dynabeads M-280 Streptavidin# (300 μL, 10 mg mL$^{-1}$) was washed by PBS several times before use to remove preservatives. The washing procedure was facilitated by using the Dynac MPC-S Magnetic Particle Concentrator. The washed Dynabeads were added to the biotin-tagged AYEMWCFHQK reaction mixture and kept at 25 °C for 30 min. The supernatant containing the unbound peptides (RPKPQQF and ASHLQLAR) was removed and labelled as the mother liquor. Beads that stuck to the wall of the Eppendorf tube were washed with $H_2O$ several times.

To cleave the peptide from the beads, PhSH (2 μL, 100 μmol mL$^{-1}$ in $CH_3CN$), DTT (5 μL, 100 μmol mL$^{-1}$ in $H_2O$) and pH 8.0 Tris-HCl/$CH_3CN$ (30 μL, 9:1) were mixed and the beads added. The reaction mixture was kept at 37 °C for 2 h. The resulting supernatant was characterised by LC–MS/MS. Recovery of AYEMWCFHQK was confirmed by LC–MS/MS.

To determine the percentage recovery of AYEMWCFHQK from the peptide enrichment experiment, a calibration curve of different concentrations of AYEMWCFHQK (125, 150, 180, 210, 240 and 300 μM) was established by LC–MS/MS. The area under the total ion count was plotted against the concentration of AYEMWCFHQK.

A 25 μL sample of the reaction mixture was injected into the HPLC apparatus. The peptide was eluted at a flow rate of 0.5 mL min$^{-1}$ with the solvent gradient shown in Figure S1 of the Supporting Information and the column effluent was monitored at 210 nm. The AYEMWCFHQK fraction was collected, freeze-dried and resuspended in $H_2O$ (10 μL). The suspension was characterised by LC–MS/MS. The area under the total ion count was obtained and compared with the calibration curve to quantify the percentage recovery of AYEMWCFHQK in the peptide enrichment experiment. The percentage recovery of native AYEMWCFHQK recovered after cleavage from the beads was found to be 71%. # Dynabeads M-280 Streptavidin was purchased from Invitrogen Dynal AS (Oslo, Norway).

**Characterisation data for 7, 8 and 9a–d**: (*Z*)-**7**: Analytical TLC (silica gel 60, 50% EtOAc in *n*-hexane): $R_f = 0.40$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta =$ 7.58–7.55 (m, 2H), 7.33–7.29 (m, 2H), 6.95 (d, $J = 9.9$ Hz, 1H), 5.90 (d, $J = 9.9$ Hz, 1H), 5.41 (br d, $J = 7.2$ Hz, 1H), 4.60–4.57 (m, 1H), 4.23 (q, $J = 7.1$ Hz, 2H), 3.27 (br d, $J = 4.3$ Hz, 2H), 1.45 (s, 9H), 1.31 ppm (t, $J =$ 7.1 Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 170.2$, 164.2, 155.1, 147.0, 137.9, 128.9, 124.1, 119.5, 116.2, 80.3, 62.1, 53.9, 38.8, 28.3, 14.1 ppm;

EIMS: $m/z$: 394 $[M]^+$; HRMS (EI): calcd for $C_{19}H_{26}N_2O_5S$: 394.1562; found: 394.1550.

(*E*)-**7**: Analytical TLC (silica gel 60, 50% EtOAc in *n*-hexane): $R_f = 0.49$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 7.59$ (d, $J = 14.8$ Hz, 1H), 7.55–7.53 (m, 2H), 7.37 (brs, 1H), 7.34–7.30 (m, 2H), 7.10 (t, $J = 7.4$ Hz, 1H), 6.08 (d, $J = 14.8$ Hz, 1H), 5.40 (brd, $J = 7.0$ Hz, 1H), 4.63–4.61 (m, 1H), 4.25 (q, $J = 7.1$ Hz, 2H), 3.32–3.24 (m, 2H), 1.46 (s, 9H), 1.31 ppm (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 170.1$, 142.6, 138.1, 129.0, 124.2, 119.8, 118.2, 80.6, 62.3, 52.9, 35.2, 28.3, 14.1 ppm; EIMS: $m/z$: 394 $[M]^+$; HRMS (EI): calcd for $C_{19}H_{26}N_2O_5S$: 394.1562; found: 394.1550.

(*Z*)-**8**: Analytical TLC (silica gel 60, 30% EtOAc in *n*-hexane): $R_f = 0.45$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 7.04$ (d, $J = 10.0$ Hz, 1H), 5.85 (d, $J = 10.0$ Hz, 1H), 5.45 (brd, $J = 7.0$ Hz, 1H), 4.60–4.56 (m, 1H), 4.23 (q, $J = 7.1$ Hz, 2H), 3.74 (s, 3H), 3.35–3.24 (m, 2H), 1.45 (s, 9H), 1.30 ppm (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 170.0$, 166.8, 155.0, 149.9, 113.4, 80.2, 62.0, 53.9, 51.3, 38.4, 28.2, 14.1 ppm; EIMS: $m/z$: 333 $[M]^+$; HRMS (EI): calcd for $C_{14}H_{23}NO_6S$: 333.1246; found: 333.1233.

(*E*)-**8**: Analytical TLC (silica gel 60, 30% EtOAc in *n*-hexane): $R_f = 0.51$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 7.59$ (d, $J = 15.3$ Hz, 1H), 5.86 (d, $J = 15.3$ Hz, 1H), 5.38 (brd, $J = 7.0$ Hz, 1H), 4.65–4.58 (m, 1H), 4.23 (q, $J = 7.1$ Hz, 2H), 3.71 (s, 3H), 3.38–3.20 (m, 2H), 1.45 (s, 9H), 1.31 ppm (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 169.9$, 146.4, 114.9, 80.5, 62.2, 53.2, 51.5, 35.3, 28.2, 14.1 ppm; EIMS: $m/z$ 333 $[M]^+$; HRMS (EI): calcd for $C_{14}H_{23}NO_6S$: 333.1246; found: 333.1233.

(*Z*)-**9a**: Analytical TLC (silica gel 60, 30% EtOAc in *n*-hexane): $R_f = 0.44$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 7.97$–7.94 (m, 2H), 7.56–7.52 (m, 1H), 7.48–7.44 (m, 2H), 7.33 (d, $J = 9.7$ Hz, 1H), 7.08 (d, $J = 9.7$ Hz, 1H), 5.45 (brd, $J = 7.2$ Hz, 1H), 4.63–4.61 (m, 1H), 4.24 (q, $J = 7.2$ Hz, 2H), 3.32 (brd, $J = 3.7$ Hz, 2H), 1.46 (s, 9H), 1.30 ppm (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (100.62 MHz, $CDCl_3$): $\delta = 189.0$, 170.1, 155.0, 152.3, 137.7, 132.5, 128.6, 128.0, 80.3, 62.1, 53.9, 39.5, 28.3, 14.1 ppm; EIMS: $m/z$: 379 $[M]^+$; HRMS (EI): calcd for $C_{19}H_{25}NO_5S$: 379.1453; found: 379.1437.

(*E*)-**9a**: Analytical TLC (silica gel 60, 30% EtOAc in *n*-hexane): $R_f = 0.53$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 7.96$ (d, $J = 8.0$ Hz, 2H), 7.83 (d, $J = 14.9$ Hz, 1H), 7.56 (t, $J = 7.4$ Hz, 1H), 7.47 (t, $J = 7.4$ Hz, 2H), 7.10 (d, $J = 14.9$ Hz, 1H), 5.41 (brd, $J = 7.0$ Hz, 1H), 4.69–4.67 (m, 1H), 4.25 (q, $J = 7.1$ Hz, 2H), 3.46–3.35 (m, 2H), 1.45 (s, 9H), 1.31 ppm (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 186.7$, 170.0, 155.0, 147.7, 137.8, 128.6, 128.4, 119.3, 80.6, 62.3, 53.2, 35.6, 28.2, 14.1 ppm; EIMS: $m/z$: 379 $[M]^+$; HRMS (EI): calcd for $C_{19}H_{25}NO_5S$: 379.1453; found: 379.1437.

(*Z*)-**9b**: Analytical TLC (silica gel 60, 40% EtOAc in *n*-hexane): $R_f = 0.47$; $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 7.27$–7.23 (m, 2H), 7.17–7.15 (m, 3H), 6.99 (d, $J = 9.7$ Hz, 1H), 6.25 (d, $J = 9.7$ Hz, 1H), 5.40 (brd, $J = 7.2$ Hz, 1H), 4.55–4.50 (m, 1H), 4.19 (q, $J = 7.1$ Hz, 2H), 3.22 (brs, 2H), 2.95–2.90 (m, 2H), 2.79–2.74 (m, 2H), 1.42 (s, 9H), 1.26 ppm (t, $J = 7.1$ Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 198.2$, 170.1, 155.1, 149.4, 145.6, 141.2, 128.5, 128.4, 126.0, 123.5, 120.3, 80.3, 62.1, 53.9, 44.5, 39.2, 31.6, 28.1, 14.2 ppm; EIMS: $m/z$: 407 $[M]^+$; HRMS (EI): calcd for $C_{21}H_{29}NO_5S$: 407.1766; found: 407.1767.

(*E*)-**9b**: Analytical TLC (silica gel 60, 40% EtOAc in *n*-hexane): $R_f = 0.50$; $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 7.54$ (d, $J = 15.3$ Hz, 1H), 7.27–7.25 (m, 2H), 7.20–7.17 (m, 3H), 6.23 (d, $J = 15.3$ Hz, 1H), 5.34 (brd, $J = 7.0$ Hz, 1H), 4.63–4.58 (m, 1H), 4.21 (q, $J = 7.2$ Hz, 2H), 3.35–3.20 (m, 2H), 2.95–2.91 (m, 2H), 2.81–2.77 (m, 2H), 1.43 (s, 9H), 1.29 ppm (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (75.48 MHz, $CDCl_3$): $\delta = 195.4$, 170.0, 155.0, 145.6, 141.1, 128.5, 128.4, 126.1, 123.5, 80.6, 62.3, 53.2, 42.7, 35.5, 31.6, 28.3, 14.1 ppm; EIMS: $m/z$: 407 $[M]^+$; HRMS (EI): calcd for $C_{21}H_{29}NO_5S$: 407.1766; found: 407.1761.

(*Z*)-**9c**: Analytical TLC (silica gel 60, 40% EtOAc in *n*-hexane): $R_f = 0.57$; $^1$H NMR (500 MHz, $CDCl_3$): $\delta = 7.94$–7.92 (m, 2H), 7.53–7.50 (m, 1H), 7.46–7.43 (m, 2H), 7.03 (s, 1H), 5.40 (brd, $J = 7.6$ Hz, 1H), 4.62–4.58 (m, 1H), 4.24 (q, $J = 7.1$ Hz, 2H), 3.44–3.30 (m, 2H), 2.63–2.60 (m, 2H), 1.65–1.56 (m, 2H), 1.48–1.40 (m, 2H), 1.44 (s, 9H), 1.28 (t, $J = 7.1$ Hz, 3H), 0.97 ppm (t, $J = 7.3$ Hz, 3H); $^{13}$C NMR (75.47 MHz, $CDCl_3$): $\delta = 188.5$, 170.2, 163.6, 155.0, 138.7, 132.1, 128.5, 128.0, 117.4, 80.3, 62.1, 53.3, 36.9, 32.5, 32.1, 28.3, 22.3, 14.1, 13.9 ppm; EIMS: $m/z$: 435 $[M]^+$; HRMS (EI): calcd for $C_{23}H_{33}NO_5S$: 435.2079; found: 435.2088.

(*E*)-**9c**: Analytical TLC (silica gel 60, 40% EtOAc in *n*-hexane): $R_f = 0.47$; $^1$H NMR (500 MHz, $CDCl_3$): $\delta = 7.97$–7.95 (m, 2H), 7.54–7.50 (m, 1H), 7.46–7.43 (m, 2H), 6.77 (s, 1H), 5.32 (brd, $J = 7.4$ Hz, 1H), 4.70–4.67 (m, 1H), 4.27–4.21 (m, 2H), 3.40–3.26 (m, 2H), 2.90–2.82 (m, 2H), 1.66–1.56 (m, 2H), 1.48–1.41 (m, 2H), 1.43 (s, 9H), 1.29 (t, $J = 7.1$ Hz, 3H), 0.94 ppm (t, $J = 7.3$ Hz, 3H); $^{13}$C NMR (75.47 MHz, $CDCl_3$): $\delta = 187.3$, 170.4, 164.6, 155.0, 139.7, 132.2, 128.5, 128.2, 113.7, 62.2, 52.4, 35.0, 33.9, 32.0, 28.3, 22.8, 14.2, 13.9 ppm; EIMS: $m/z$: 435 $[M]^+$; HRMS (EI): calcd for $C_{23}H_{33}NO_5S$: 435.2079; found: 435.2078.

(*Z*)-**9d**: Analytical TLC (silica gel 60, 40% EtOAc in *n*-hexane): $R_f = 0.60$; $^1$H NMR (500 MHz, $CDCl_3$): $\delta = 7.29$–7.26 (m, 2H), 7.18–7.16 (m, 3H), 6.23 (s, 1H), 5.34–5.31 (m, 1H), 4.57–4.54 (m, 1H), 4.23 (q, $J = 7.1$ Hz, 2H), 3.37–3.23 (m, 2H), 2.95–2.92 (m, 2H), 2.78–2.74 (m, 2H), 2.46–2.43 (m, 2H), 1.52–1.48 (m, 2H), 1.44 (s, 9H), 1.39–1.33 (m, 2H), 1.29 (t, $J = 7.1$ Hz, 3H), 0.93 ppm (t, $J = 7.3$ Hz, 3H); $^{13}$C NMR (75.47 MHz, $CDCl_3$): $\delta = 197.3$, 170.2, 160.6, 141.5, 128.5, 128.4, 126.0, 120.5, 62.1, 53.3, 44.8, 36.2, 32.3, 31.9, 30.4, 29.7, 28.3, 22.2, 14.2, 13.8 ppm; EIMS: $m/z$: 463 $[M]^+$; HRMS (EI): calcd for $C_{25}H_{37}NOS$: 463.2392; found: 463.2396.

(*E*)-**9d**: Analytical TLC (silica gel 60, 40% EtOAc in *n*-hexane): $R_f = 0.62$; $^1$H NMR (500 MHz, $CDCl_3$): $\delta = 7.29$–7.26 (m, 2H), 7.21–7.18 (m, 3H), 6.01 (s, 1H), 5.26–5.22 (m, 1H), 4.57–4.54 (m, 1H), 4.22 (q, $J = 7.1$ Hz, 2H), 3.24–3.08 (m, 2H), 3.04–2.98 (m, 2H), 2.94–2.88 (m, 2H), 2.76–2.72 (m, 2H), 1.52–1.48 (m, 2H), 1.44 (s, 9H), 1.32–1.24 (m, 5H), 0.94–0.86 ppm (m, 3H); $^{13}$C NMR (75.47 MHz, $CDCl_3$): $\delta = 195.6$, 170.4, 162.9, 141.4, 128.4, 128.3, 126.0, 116.3, 61.9, 52.2, 46.0, 34.5, 33.8, 31.9, 30.4, 29.7, 27.4, 22.7, 14.2, 13.9 ppm; EIMS: $m/z$: 463 $[M]^+$; HRMS (EI): calcd for $C_{25}H_{37}NOS$: 463.2392; found: 463.2390.

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