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Three-Component Protein Modification Using Mercaptobenzaldehyde Derivatives

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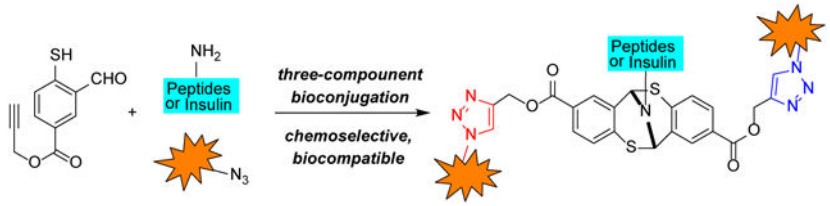
Abstract

A chemoselective primary amine modification strategy which enables the three-component, one-pot bioconjugation is described. The specifically designed, mercaptobenzaldehyde-based bifunctional linker achieves highly selective and robust amine labeling under biocompatible conditions. This linker demonstrates wide functional group tolerance and is simple to prepare which allowed facile payload incorporation. Finally, our studies have shown that the introduction of linker does not impair the function of modified protein such as insulin.

Graphical Abstract



Efficient chemical modification of peptide or protein systems with multiple components has been shown to be essential towards achieving important biological and therapeutic functions.[1] A number of therapeutic applications have recently been reported where proteins have been functionalized with different bioactive components.[2] As the case in point, the usage of multiple antigen modified trivalent peptides to trigger a potent immune response,[3] linkage of polypeptide and biotin in a three-component fashion to facilitate elucidation of intracellular interactome with pull-down assays.[4,5] Despite the aforementioned examples,

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Supporting Information

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Complete experimental procedures and characterization data for all new compounds (PDF)

Accession Codes

CCDC 1825270 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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convergent synthetic strategies to achieve multi-component peptide assemblies in a one-pot reaction remain challenging.

A number of approaches have emerged in recent years.[6] Most notably, Francis et al. reported a three-component Mannich-type transformation for selective tyrosine modification.[7] Meanwhile, Ball and co-workers recently described an organometallic approach for three-component tyrosine bioconjugation.[8] Herein, we report a one-pot three-component protocol that is specifically targeting primary amine position at native polypeptides and proteins under mild biocompatible conditions. The described approach builds upon the conventional 'click' modification[9] (Figure 1a) and recently developed bioorthogonal strategies that entail genetic incorporation of unnatural amino acid residues and typically result in mono-functionalized peptides.[10]

Our approach is based on the our understanding of the unique reactivity of the thiol group towards imines.[11] We hypothesized that alkynyl mercaptobenzoate **1** will be a suitable heterobifunctional linker which can be concisely synthesized (Figure 1b). The mercaptobenzoate end of the molecule will selectively react with primary amines whereas alkynyl group could facilitate a robust copper-catalyzed azide alkyne cycloaddition (CuAAC)[12] within the protein framework. The proposed chemoselective amine conjugation requires no exogenous additives or reagents, concurrent conjugation of primary amines and CuAAC may be carried out at the same time in a one-pot manner. Lastly, due to the planar structure of aryl ring, the shown modification is unlikely to impair the native functions and activity of the targeted peptides or proteins.[13]

To test our hypothesis, we carried out a model study shown in Scheme 1. A known disulfide substrate **2** was obtained from the commercially available mercapto-benzyl alcohol in two steps. The reaction of **2** and glycine methyl ester under the conditions (TCEP, THF/PBS buffer (1:9)) at neutral pH (6.8) generated **4** containing a unique [3.3.1] bicyclo nonane structure in 90% yield. Previously, compound **4** has been reported as the analogue of Tröger's base.[14] The pioneering work by Toste and co-workers described synthesis of **4** in non-aqueous solution and at the elevated temperature (80 °C).[15] We found that the transformation could be carried out at room temperature and in a biocompatible PBS buffer (Scheme 1). We believe that the reaction began with reduction of the disulfide bond of **2** to liberate mercaptobenzaldehyde **5**. Amination of **5** with the glycine methyl ester **3** generated imine **6** which was trapped by a second equivalent of **5**. The adduct **7** subsequently underwent intramolecular amination and yielded imine **8**. Another intramolecular thiol addition to the iminium intermediate afforded bicyclic product **4**, which was confirmed by X-ray analysis.

Intrigued by the benign reaction conditions and excellent yields, we hypothesized that the mercaptobenzaldehyde derivatives could be applied toward the modification of primary amines within the protein's framework. However, utilization of the disulfide **2** would not be ideal. The substrate would require disulfide reduction using TCEP which could also disrupt the protein's disulfide linkages, thereby denaturing the protein. On the other hand, a motif with a similar structure of the substrate **5** would be preferred. Addition of an alkynyl group facilitates a secondary site of conjugation using well-established CuAAC chemistry.

Synthesis of the heterobifunctional linker was carried out in two steps using readily available 4-bromo-3-formylbenzoic acid **9**, as shown in Table 1. *O*-propargylation furnished bromo-benzaldehyde **10** in 95% yield, which was treated with sodium sulfide in dimethylformamide afforded **1** in 90% yield.

With the desired compound in hand, we next investigated the reactivity of **1** in the bioconjugation settings. Under the standard reaction conditions, the coupling of linker **1** and peptide proceeded smoothly and the bicyclic adduct **11** was produced in high yield in THF/PBS buffer. The small amount of THF was necessary to achieve complete solubility of **1**. A small library of peptides was prepared and subjected to the standard bioconjugation conditions (Table 1). It is notable that the described chemistry is compatable with functional groups that are relevant to proteins and polypeptide. Fmoc protected peptides **11a** furnished lysine modification products in 83% yield. Hydrophobic and hydrophilic sidechains are well tolerated; lysine could be modified in the presence of adjacent carboxylic acid or amide groups. Secondary amine of proline *N*-terminus will not engage bioconjugation and lysine was modified with excellent yields (**11b**-**11g**). We next examined the chemoselectivity between peptidyl *N*-terminus and the lysine ε-amino group (entries **11h**-**11j**). Under neutral reaction medium, lysine was selectively modified when the polypeptides constitute sterically demanding amino acids such as valine residues at the *N*-terminus. The polypeptides bearing either alanine or glycine residues at the *N*-terminus can also be efficiently functionalized with compound **1** (entries **11k**-**11n**). Overall, the linker **1** demonstrated an exclusive preference for primary amines in the polypeptides. The reaction conditions are compatible with all type of amino acid residues. Commonly known nucleophilic peptidyl sidechains such as His, Cys, Trp, and Ser are compatible with our bioconjugation approach. Other amino acids with polar sidechains (Asn, Glu, Tyr, Met and Arg) would not interfere with the described conjugations.

We subsequently investigated one-pot three-component bioconjugation using compound **1** under the CuAAC conditions ($CuSO_4$, sodium ascorbate, TBTA, *t*-BuOH/H_2O (1:1)). The reaction involving linker **1**, glycine methylester **3**, and the azido cargos **12** generated the double-clicked adducts **13**. In one step, through linker **1** and produced **13a** and **13b** in 87% and 86% yields respectively. Fluorophore azido-methoxycoumarin and benzyl azide and acetophenone azide were connected to glycine monosaccharide azides were evaluated, and **13c**-**13e** were obtained in good to excellent yield.

We have discovered that the level of cargo installation can be controlled using azido compounds as limiting reagents. The one-pot reaction exclusively yielded double-clicked products when the 2.4 equivalents of azido compounds were used (**13a**-**13e**) (Scheme 2a). On the other hand, when 1.2 equivalents of azido compounds were used, the mono-clicked adducts were produced (Scheme 2b). Under the mono-clicked conjugation conditions, fluorophore azido-methoxycoumarin was furnished in 72% yield (**15b**). In addition, we evaluated a variety of azides, such as monosaccharide azides (**15d**-**15e**), and a biotinylation reagent azide-PEG3-biotin (**15f**). these examples successfully modified target amino acids or peptides in good yields in a one-pot fashion. Moreover, one-pot labeling experiments were extended to polypeptides without the erosion of the yields. Methoxycoumarin and glucose was connected to the peptides in good yields under bioconjugation conditions (**15h**-**15j**).

Furthermore, mono-clicked constructs generated in Scheme 2b retained an unreacted alkynyl group which potentially could be used to intergrade a different labeling group. We continued with our investigation with these considerations (Scheme 3). A consequent conjugation of mono-functionalized compounds **15c**-**15e** was achieved under standard reaction conditions. Formation of the heterobifunctional compounds **16** was realized. The conjugate successfully placed both *N*-acetylglucosamine and methoxycoumarin on the molecule in excellent yield (**16a**). Glucose, benzylphenone and methoxycoumarin were combined to furnish **16b** and **16c** with almost quantitative yields. The generation of **16d** required longer reaction time and provided an 85% yield. In principle, the highly diversified functional groups could be introduced to the amino sites of the protein.

To demonstrate the practicality of our three-component bioconjugation strategy, we performed the modification of a more sophisticated protein. The recombinant human insulin was selected due to its well-established biological activities. Structurally, insulin consists of two peptide chains, the 21 amino acid A chain and the 30 amino acid residue B chain which are connected by three pairs of disulfide bonds. Lys residue is located at B53. The *N*-terminus of insulin was chosen for modification, as it would cause the least perturbation to its activity.[13] Considering the insulin could form insoluble fibrils upon stirring,[16] the optimized conditions were used to chemoselectively modify insulin at the *N*-terminus (Figure 2a). The linkage between insulin and AF546 azide was accomplished in one-pot setting. The modification of the insulin site specifically occurred at the both *N*-termini of A chain and B chain, which was validated by the extensive LC-MS/MS analysis.[17] The N-termini selectivity might be attributed to either the lower pKa of N-terminus compared to lysine amino group or the steric of insulin shielding the lysine side chain.[16,18] More interestingly, the disulfide bonds remained intact during the modification process which suggested that our linker is compatible with commonly observed protein disulfide linkages.

As illustrated in Figure 2c, the modified insulin is well-suited for cell biology studies. Labeling of insulin with the AF-546 fluorophore showed no obvious perturbation to the insulin's functions. THP1 cells incubated with the modified insulin show the expected strong fluorescence, whereas the control cells incubated with the fluorophore and native insulin demonstrated negative fluorescence. Further examination of the fluorescent cells found that fluorescence is not uniform across the cells but rather forms clusters near cellular nuclei. That might be due to the rapid internalization of insulin-receptor being transported to endosomal apparatus of the cell.[19] Such a phenomenon implies that the insulin activity was not impaired by the conjugation.

In summary, we have established a general protocol for chemo-selective conjugation of primary amine groups within the protein framework using alkynyl mercaptobenzaldhyde **1**. This bioorthogonal method allows native peptide and protein conjugation with two different functional groups, thus facilitating rapid modification of biomolecules with great flexibility. We have shown that the method is compatible with a variety of amino acid side-chains and disulfide linkages, which allows facile attachment of functional payloads, such as glycans, fluorescent groups and affinity handles in a one-pot fashion. Moreover, the modification of the mercaptobenzaldhyde aryl ring could introduce a variety of moieties other than alkynyl ester, and it is reasonable to speculate that diversified functional groups[20] could be selected

as the suitable linkers. The practicality of our approach was illustrated by modifying insulin with fluorescent groups and carried out cell imaging experiments which suggested that the protein's functions were not impaired. Further investigations of the bifunctional linker towards other protein modification will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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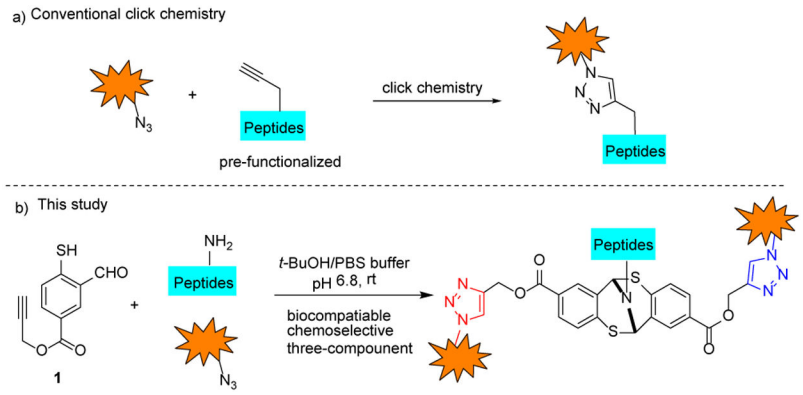


Figure 1.
Three-component one-pot bioconjugation.

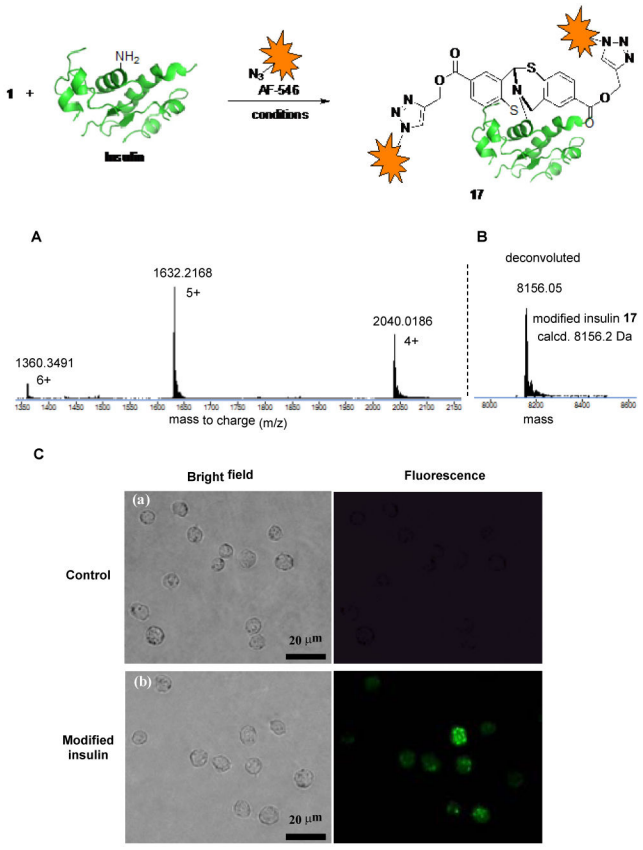
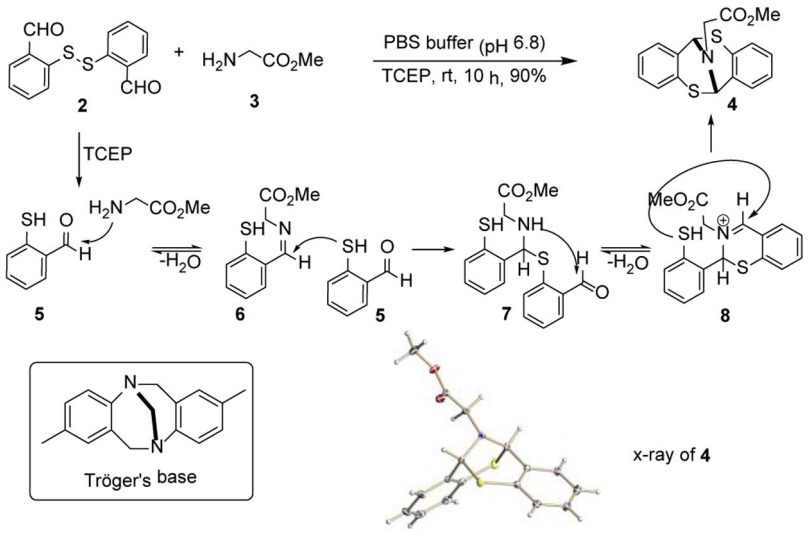


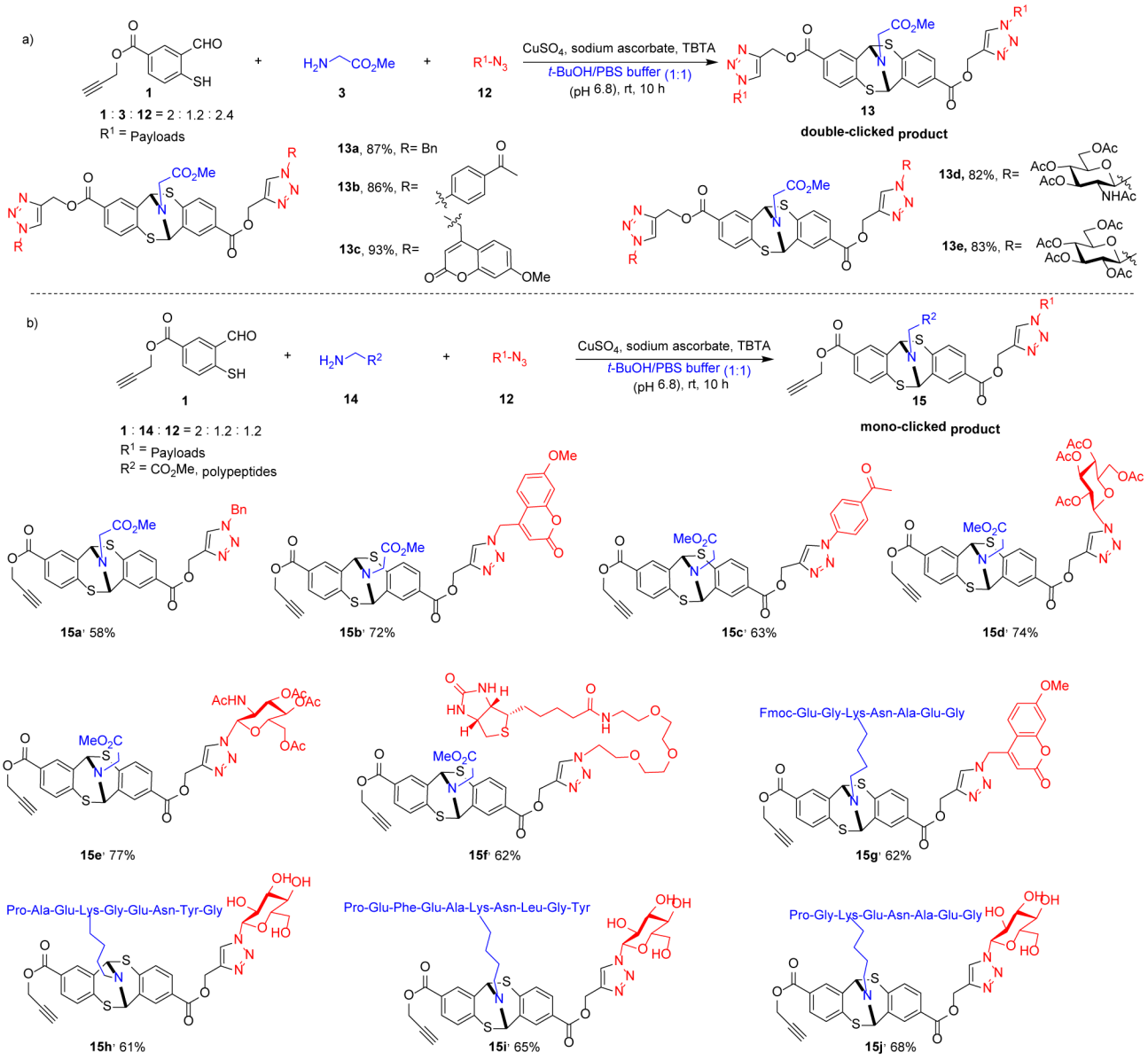
Figure 2.

Three-component conjugation of human insulin. Reagents and conditions: 100 μL THF/PBS (1:4, v/v), pH 5.5, 0.15 equivalent of $CuSO_4$, 0.3 equivalent of sodium ascorbate, 0.3 equivalent of TBTA and 9 equivalents of AF546 azide were used, final concentration of insulin is 1.72 mM, incubated for 4 h. Examination of modified insulin **17** function on THP1 cells. (a) Bright field and fluorescence images of THP1 cells incubated with 0.06 mM AF546 azide for 4 h. (b) Bright field and fluorescence images of THP1 cells incubated with 0.06 mM modified insulin **17** for 4 h. Scale bar: 20 μm.



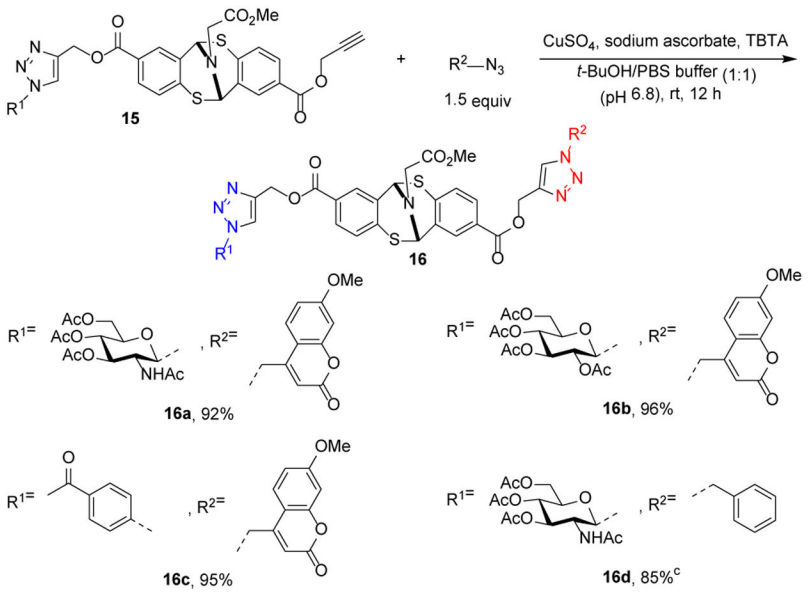
Scheme 1. Synthesis of bicyclic [3.3.1] framework under mild conditions.[a]

[a] Reaction conditions: compound **2** (0.364 mmol) was dissolved in THF (0.4 mL), THF/PBS buffer (1: 9, v/v), rt, 10 h.



Scheme 2. One-pot bioconjugation with different azido compounds.[a, b]

[a] Reaction conditions: 0.1 equivalent of $CuSO_4$, 0.2 equivalent of sodium ascorbate, 0.1 equivalent of TBTA and 2 equivalents of **1**, 1.2 equivalent of glycine methyl ester hydrochloride and 1.2 or 2.4 equivalent of azide were used. [b] Isolated yields.

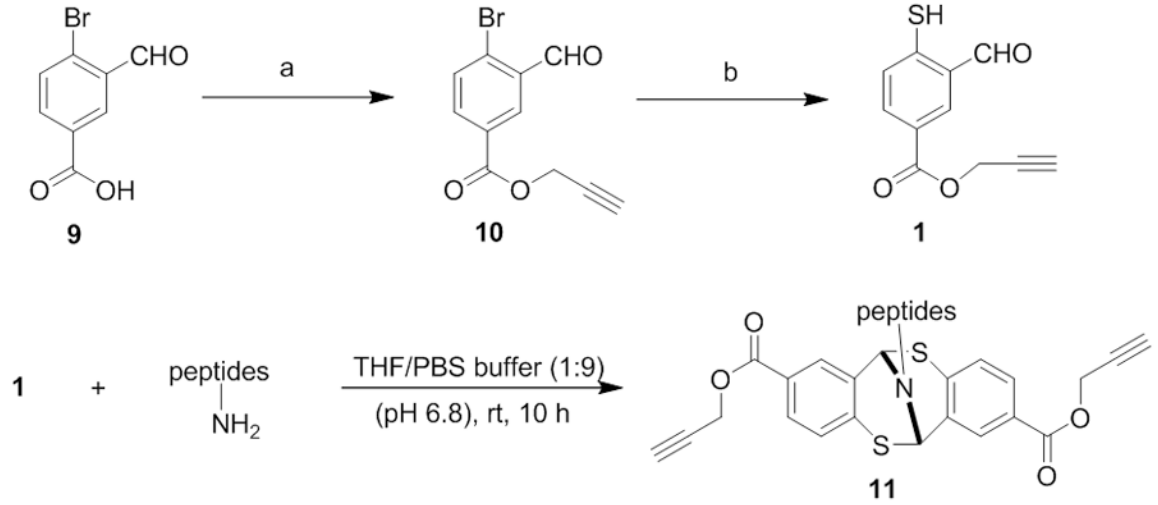


Scheme 3. Second cargo incorporation of mono-clicked products.[a, b]

[a] Reaction conditions: 0.1 equivalent of $CuSO_4$, 0.2 equivalent of sodium ascorbate, 0.1 equivalent of TBTA and 1.5 equivalents of azido compound were used. [b] Isolated yields. [c] Reaction was stirred for 30 h.

Table 1.

Scope of peptides in conjugation with bifunctional linker **1**.



	peptides	yields[c]		peptides	yields
11a	**Fmoc-Glu-Gly-Lys-Asn-Ala-Glu-Gly**	83%	**11b**	**Pro-Ala-Lys-Met-Gln-His-Gly**	88%
11c	**Pro-Glu-Asn-Leu-Lys-Tyr**	90%	**11d**	**Pro-Tyr-Gly-Lys-Gln-Leu-Glu-Gly**	84%
11e	**Pro-Gly-Lys-Glu-Asn-Ala-Glu-Gly**	90%	**11f**	**Pro-Ala-Glu-Lys-Gly-Glu-Asn-Tyr-Gly**	86%
11g	**Pro-Glu-Phe-Glu-Ala-Lys-Asn-Leu-Gly-Tyr**	88%	**11h**	**Val-Glu-Lys-Gln-Ile-Asn-Tyr**[d]	82%
11i	**Val-Glu-Ala-Lys-Asn-Leu-Glu-Gly**[d]	78%	**11j**	**Val-Leu-Glu-Lys-Asn-Gly-Glu-Gly-Gln-Tyr**[d]	71%
11k	**Ala-Gln-Ser-Gly-Cys-Glu-Gly**	85%	**11l**	**Ala-Gln-Leu-Glu-Tyr**	81%
11m	**Gly-Tyr-Glu-Ala-Gln-Gly**	75%	**11n**	**Gly-Glu-Ala-Trp-Leu-Arg-Gly**	89%

*[a]*Reaction conditions: propargyl bromide, K_2CO_3, DMF, rt, 2 h, 95%

*[b]*Reaction conditions: Na_2S, DMF, rt, 5 h, 90%. Compound **1** was dissolved in in THF (80 μL), THF/PBS buffer (1: 9, v/v), final concentration of peptides and compound **1** is 0.01 M and 0.022 M.

*[c]*Isolated yields.

*[d]*Structure was confirmed by tandem MS spectrum.