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Three-Component Protein Modification Using Mercaptobenzaldehyde Derivatives

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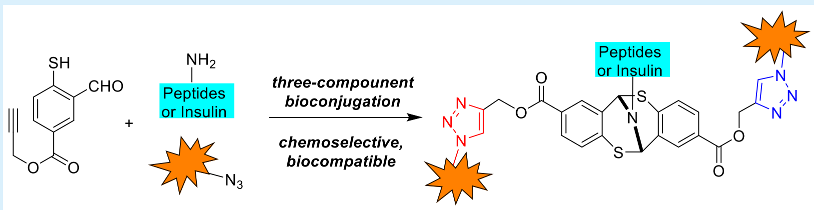
Supporting Information



ABSTRACT: A chemoselective primary amine modification strategy that 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.

Efficient chemical modification of peptide or protein systems with multiple components has been shown to be essential toward achieving important biological and therapeutic functions.[1] Several 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 antigens 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, convergent synthetic strategies to achieve multicomponent peptide assemblies in a one-pot reaction remain challenging.

Many 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] (see Figure 1a) and recently developed bioorthogonal strategies that entail genetic incorporation of unnatural amino acid residues and typically result in monofunctionalized peptides.[10]

Our approach is based on the understanding of the unique reactivity of the thiol group toward imines.[11] We hypothesized that alkynyl mercaptobenzoate **1** will be a suitable hetero-bifunctional linker, which can be concisely synthesized (see Figure 1b). The mercaptobenzoate end of the molecule will

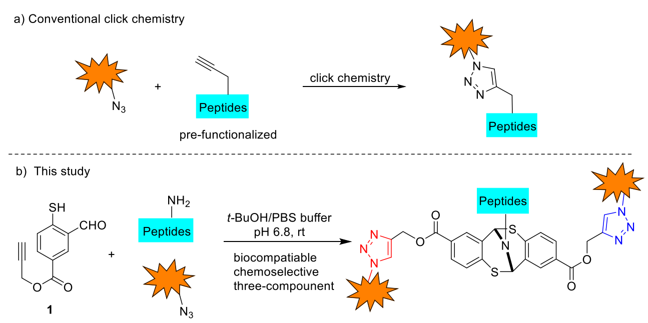


Figure 1. Three-component one-pot bioconjugation: (a) conventional click chemistry and (b) this study.

selectively react with primary amines, whereas an 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 performed at the same time in a one-pot manner. Lastly, because of the planar structure of aryl ring, the modification shown is unlikely to impair the native functions and activity of the targeted peptides or proteins.[13]

To test our hypothesis, we performed a model study that is depicted in Scheme 1. A known disulfide substrate (**2**) was obtained from the commercially available mercapto-benzyl

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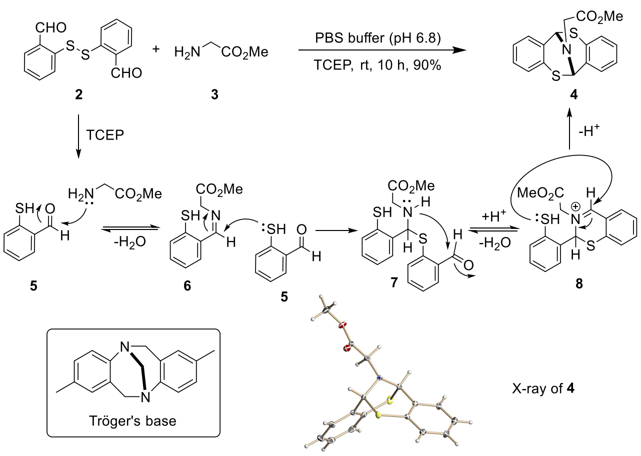
Scheme 1. Synthesis of Bicyclic [3.3.1] Framework under Mild Conditions[a]



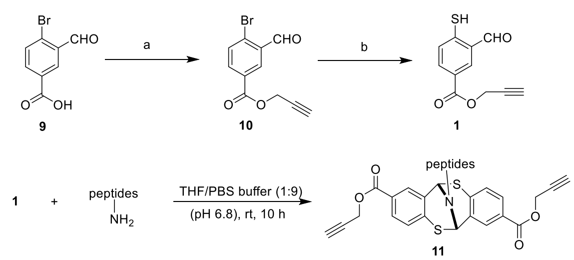
[a]Reaction conditions: compound **2** (0.364 mmol) was dissolved in THF (0.4 mL), THF/PBS buffer (1:9, v/v), room temperature (rt), 10 h.

alcohol in two steps. The reaction of **2** and glycine methyl ester under conditions of TCEP, THF/PBS buffer (1:9, v/v) 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 nonaqueous solution and at the elevated temperature (80 °C).[15] We found that the transformation could be performed 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 mercapto-benzaldehyde **5**. Amination of **5** with 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 iminium ion **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 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 substrate **5** would be preferred. The addition of an alkynyl group facilitates a secondary site of conjugation using well-established CuAAC chemistry. Synthesis of the heterobifunctional linker was performed 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 (see Table 1). It is notable that the described chemistry is compatible with functional groups that are relevant to proteins and polypeptide. Fmoc-protected peptides (**11a**) furnished lysine modification products in 83% yield. Hydrophobic and hydrophilic side chains 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 a 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**)

Table 1. Scope of Peptides in Conjugation with Bifunctional Linker 1*



	peptide	yield** (%)		peptide	yield** (%)
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-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-lle-Asn-Tyr***	82
11i	Val-Glu-Ala-Lys-Asn-Leu-Glu-Gly***	78	**11j**	Val-Leu-Glu-Lys-Asn-Gly-Glu-Gly-Gln-Tyr***	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

*Reaction conditions for (a): propargyl bromide, K_2CO_3, DMF, rt, 2 h, 95%. Reaction conditions for (b): 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 and 0.022 M. **Isolated yields. ***Structure was confirmed by tandem MS.

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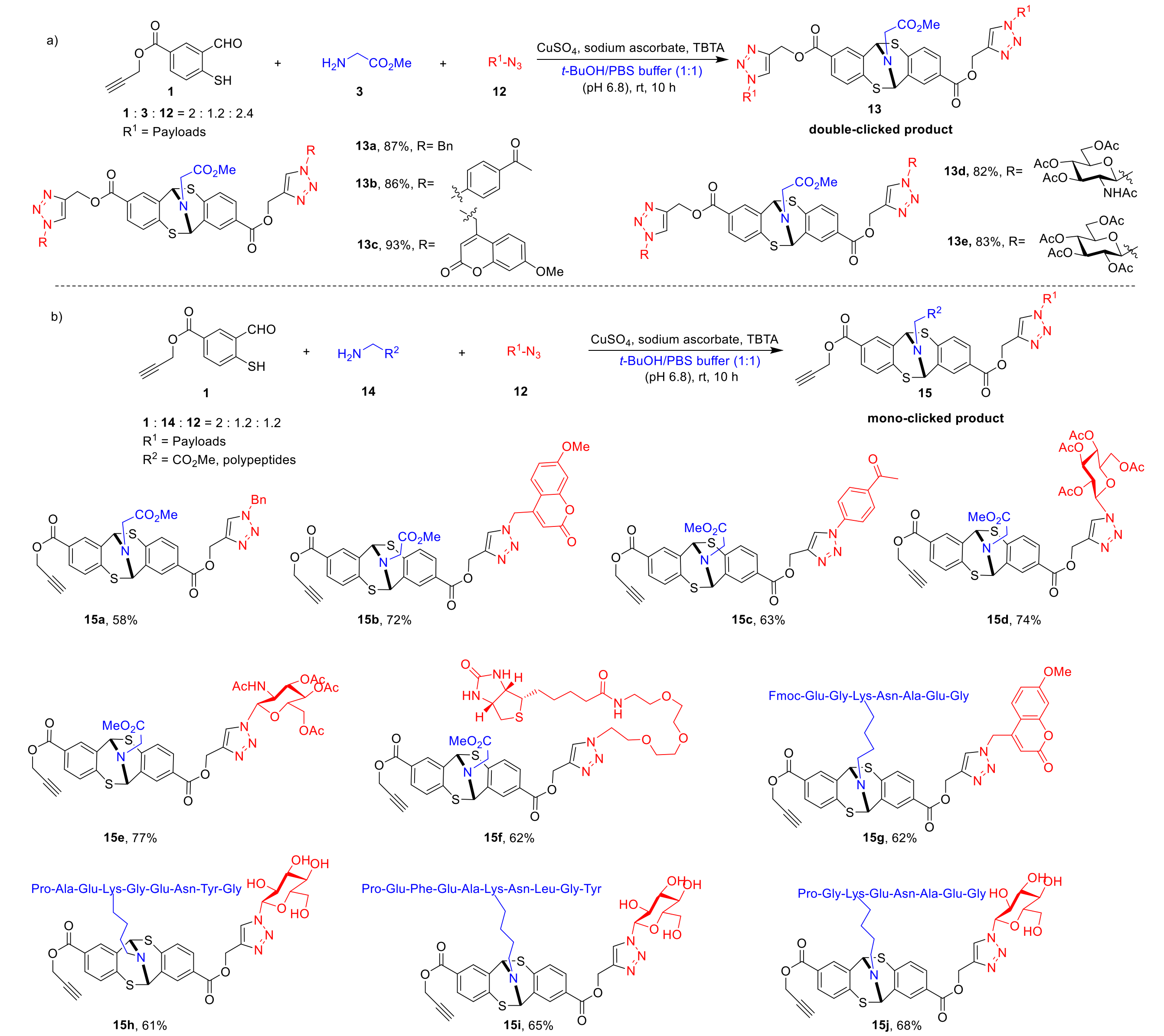
Scheme 2. One-Pot Bioconjugation with Different Azido Compounds*



*Reaction conditions: 0.1 equiv of $CuSO_4$, 0.2 equiv of sodium ascorbate, 0.1 equiv of TBTA and 2 equiv of **1**, 1.2 equiv of glycine methyl ester hydrochloride and 1.2 or 2.4 equiv of azide were used. Isolated yields were shown in all examples.

demonstrated an exclusive preference for primary amines in the polypeptides. The reaction conditions are compatible with all types of amino acid residues. Commonly known nucleophilic peptidyl side chains such as His, Cys, Trp, and Ser are compatible with our bioconjugation approach. Other amino acids with polar side chains (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, benzyl azide and acetophenone azide were connected to glycine through linker **1** and produced **13a** and **13b** in 87% and 86% yields, respectively. Fluorophore azido-methoxycoumarin and monosaccharide azides were also 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 2.4 equiv of azido compounds were used (**13a**−**13e**) (Scheme 2a). On the other hand, when 1.2 equiv of azido compounds were used, the monoclicked adducts were produced (Scheme 2b). Under the monoclicked 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

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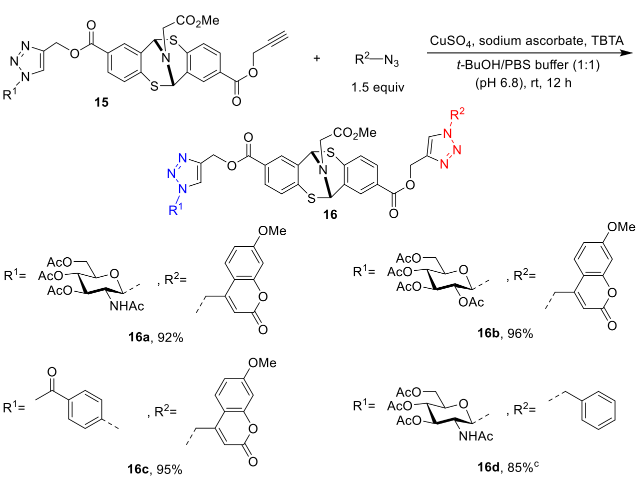
connected to the peptides in good yields under bioconjugation conditions (**15h**−**15j**).

Furthermore, monoclicked constructs generated in Scheme 2b retained an unreacted alkynyl group which potentially could be used to intergrade a different labeling group. We continued our investigation with these considerations (see Scheme 3). A

Scheme 3. Second Cargo Incorporation of Monoclicked Products[a,b]



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

consequent conjugation of monofunctionalized 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 because of 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 the 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 pK_a of N-terminus, compared to the 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

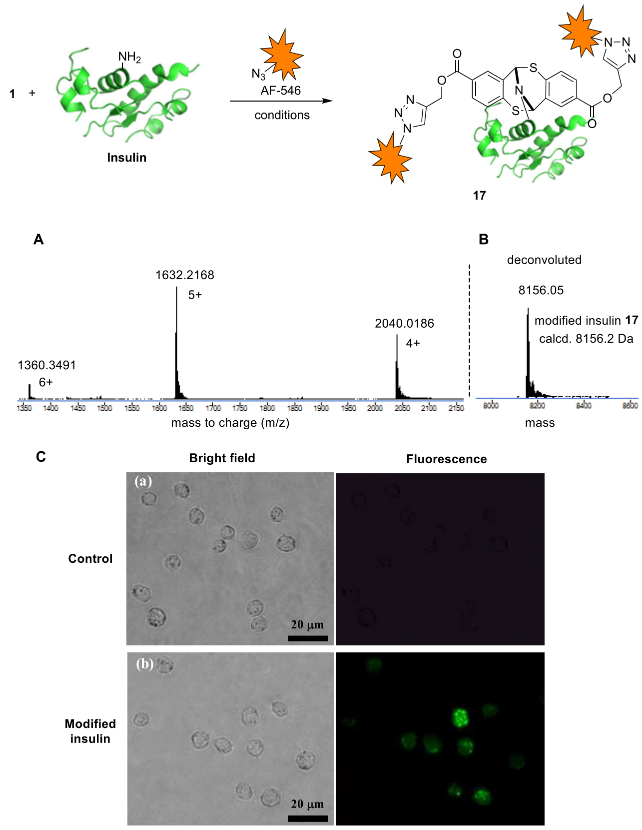


Figure 2. (A) Three-component conjugation of human insulin. Reagents and conditions: 100 μL THF/PBS (1:4, v/v), pH 5.5, 0.15 equiv of $CuSO_4$, 0.3 equiv of sodium ascorbate, 0.3 equiv of TBTA and 9 equiv of AF546 azide were used, final concentration of insulin is 1.72 mM, incubated for 4 h. (B) Examination of modified insulin **17** function on THP1 cells. Panel (C) shows (a) bright-field and fluorescence images of THP1 cells incubated with 0.06 mM AF546 azide for 4 h and (b) bright-field and fluorescence images of THP1 cells incubated with 0.06 mM modified insulin **17** for 4 h. Scale bar = 20 μm.

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, instead, forms clusters near cellular nuclei. That might be due to the rapid internalization of the insulin receptor being transported to the 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 chemoselective conjugation of primary amine groups within the protein framework using alkynyl mercaptobenzaldehyde (**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

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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 cell imaging experiments were performed, which suggested that the protein's functions were not impaired. Further investigations of the bifunctional linker toward other protein modification will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01294.

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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Notes

The authors declare no competing financial interest.

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