

Selective Modification of Alkyne-linked Peptides and Proteins by Cyclometalated Gold(III) (C^N) Complex-mediated Alkynylation

Author

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Abstract

Alkyne is a useful functionality incorporated in proteins for site-selective bioconjugation reactions. Although effective bioconjugation reactions such as copper(I)-catalyzed and/or copper-free 1,3-dipolar cycloadditions of alkynes and azides are the most common approaches, the development of new alkyne-based bioconjugation reactions is still an ongoing interest in chemical biology. In this work, a new approach has been developed for selective modification of alkyne-linked peptides and proteins through the formation of arylacetylenes by a cross-coupling reaction of 6-membered ring cyclometalated gold(III) (C^N) complexes (HC^N = 2-arylpyridines) with terminal alkynes. Screening of the reaction conditions with a series of cyclometalated gold(III) complexes with phenylacetylene gave an excellent yield (up to 82%) by conducting the reaction in slightly alkaline aqueous conditions. The reaction scope was expanded to various alkynes, including alkyne-linked peptides to achieve up to >99% conversion. Using fluorescent dansyl (**11**) and BODIPY (**1m**)-linked gold(III) complexes, alkyne-linked lysozyme has been selectively modified.

Introduction

Selective modification of biomolecules has become an important tool for the development of novel bioconjugates which are essential for biological studies.¹ Although significant advancements have been made in chemical synthesis, only a limited number of chemical reactions can be applied on selective modification of biomolecules due to the stringent requirements of high reaction efficiency, excellent selectivity and mild reaction conditions (aqueous medium, temperature around 40 °C and near neutral pH).² Thus, it remains a continuous interest on the development of novel and efficient methods for selective modification of peptides and proteins in aqueous medium.

Classical protein modifications mainly focus on bioconjugation of lysine and cysteine due to the high nucleophilicity of these amino acid residues.^{1a,2} Treated with N-hydroxysuccinimide (NHS) esters, lysine can be conveniently modified through acylation. However, the prevalence of lysine residues on protein surfaces may result in non-selective multiple modifications leading to poor regioselectivity in the bioconjugation. Cysteine with high reactivity has become an attractive amino acid residue for modification. However, the drawbacks of cysteine bioconjugation include its relatively sparse occurrence (1.7%) in proteins and hence the need of introduction by site-directed mutagenesis.

Incorporation of bioconjugation handles (alkynes and azides) by chemical tagging or genetic manipulation followed by selective modification of the handles via bioconjugation chemistry has become an emerging area for site selective modification of proteins.³ Introduction of alkynes onto protein surface which allows “click chemistry” via copper-catalyzed 1,3-dipolar cycloaddition of alkynes and azides is one of the most accessible approaches.⁴ Besides, Lin *et al.* have reported examples of modification of alkyne-linked proteins using palladium(II) complexes.⁵ Apart from these advances, the development of novel approaches for selective modification of alkyne-linked proteins still remains a significant interest in chemical biology research.

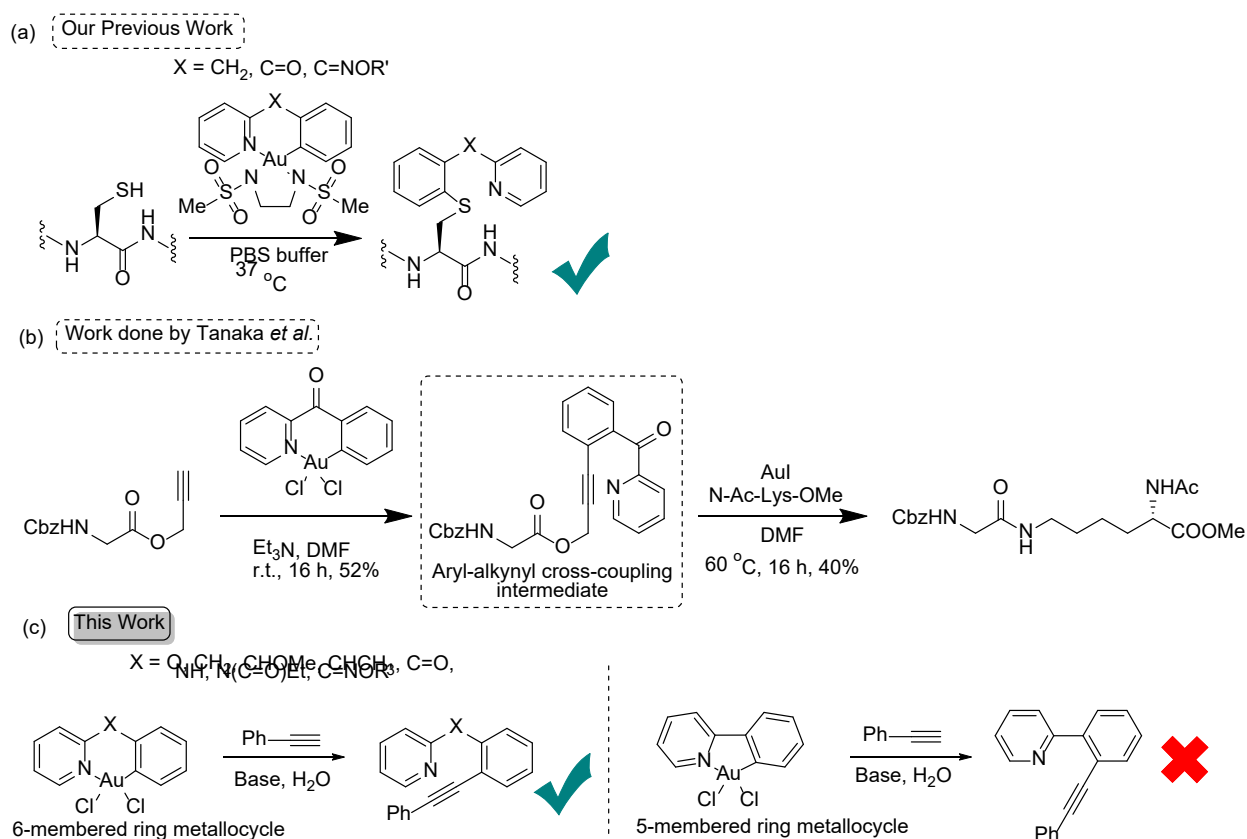
Apart from traditional organic bioconjugation methods, chemoselective, site-specific and spatially resolved modification of peptides and proteins can also be achieved by the use of transition-metal complexes which show unique fundamental reactivity, rich morphological information and diverse ligand-controlled coordination geometry that provide new applications in preparation of protein therapeutics, biomaterials and chemical biology.⁶ Among those advances, significant breakthroughs include the employment of rhodium(II)-carbenoids for tryptophan and

cysteine modifications by Francis⁷ and Ball,⁸ as well as using organopalladium(II) species for modification of cysteine, lysine as well as alkyne handles by Lin,⁵ Pentelute and Buchwald⁹.

Cyclometalated gold(III) complexes with interesting catalytic, biological and photophysical properties have been applied in various research fields.¹⁰⁻¹⁶ Along with our research efforts on gold catalysis^{11c,11d,11f,11g,12} and bioconjugation¹⁷, in 2014, we first reported a cysteine modification via ligand-controlled C-S bond formation using cyclometalated gold(III) (C[^]N) complexes [Au(C[^]N)msen] (HC[^]N = 2-arylpyridines; msen = *N,N'*-bis(methanesulfonyl) ethylenediamine) (Scheme 1a).¹⁸ We found that 6-membered ring cyclometalated gold(III) (C[^]N) complexes were much more efficient than the 5-membered ring analogues to form the C-S bond with thiol-containing compounds. Recently, arylation in peptides and proteins using organometallic gold(III) reagents has been demonstrated to become as efficient as the promising palladium(II)-mediated approaches.¹⁹ Besides, Tanaka *et al.* have developed a cyclometalated gold(III) complex-mediated propargyl ester amidation for *in vivo* protein labelling on the lysine residues.²⁰ A further study indicated the reductive elimination product between the 2-benzoylpyridine ligand (Hpcp) and the propargyl ester was a crucial intermediate in the sequential amidation reaction (Scheme 1b).^{20b} However, in comparison to the success in cysteine and lysine modification, employment of cyclometalated gold(III) complexes for alkyne-linked peptide or protein modification still remains sparse.

Inspired by the recent advances in gold catalysis which indicates the rapid C-C bond formation reactions by reductive elimination of gold(III) species²¹ and the key aryl-alkynyl cross-coupling intermediate isolated by Tanaka,^{20b} we set out to use the organometallic gold(III) species for modification of alkyne-linked peptides and proteins. Herein, we report a new approach for labelling terminal alkyne-linked peptides and proteins via ligand-controlled alkynylation with 6-

membered ring cyclometalated gold(III) complexes $[\text{Au}(\text{C}^{\wedge}\text{N})\text{Cl}_2]$ (Scheme 1c). Cross-coupling of the 2-arylpyridine analogues of 6-membered ring cyclometalated gold(III) complexes with terminal alkynes has been demonstrated to proceed smoothly under mild reaction conditions in aqueous medium. This newly developed cyclometalated gold(III)-mediated alkylation has been applied on selective modification of alkyne-linked peptides with excellent conversions. Using fluorescent dye-linked gold(III) complexes, fluorescent-labelling of alkyne-containing lysozyme has also been used to demonstrate the chemoselectivity of this method.



Scheme 1. (a) Cysteine modification by $[\text{Au}(\text{C}^{\wedge}\text{N})\text{msen}]$ complexes via C–S bond formation. (b)

Cyclometalated gold(III) complex $[\text{Au}(\text{pcp})\text{Cl}_2]$ -mediated propargyl ester amidation. (c)

Alkylation by $[\text{Au}(\text{C}^{\wedge}\text{N})\text{Cl}_2]$ complexes via C–C bond formation.

Results and Discussion

Cyclometalated gold(III) complexes $[\text{Au}(\text{C}^{\wedge}\text{N})\text{L}_2]$ **1a-1k** (L = chloride or 4-fluorophenylacetylene) with 6- or 5-membered ring metallocycles were synthesized based on literatures and our previous works (Figure 1).^{11d,12b,17d,18} To model the reaction and optimize the reaction conditions, we found that alkynylated 2-phenoxy pyridine **3a** was obtained as an expected product by treatment of $[\text{Au}(\text{pop})\text{Cl}_2]$ **1a** (Hpop = 2-phenoxy pyridine) with phenylacetylene **2a**. Using 1 equivalent of **2a** gave 40% yield of **3a** in the presence of 5 equivalents of NaHCO_3 at 40 °C (Table 1, entry 1). Increasing the amount of **2a** facilitated the formation of **3a** up to 74% yield (Entries 2-3). Using either acetonitrile (CH_3CN) or H_2O as solvent slightly decreased the yields (Entries 4-5). The yield was improved to 82% by using K_2CO_3 as the base (Entry 6). Using organic base such as triethylamine (Et_3N) did not improve the yield (66%) (Entry 7). Addition of triphenylphosphine (PPh_3) which is supposed to facilitate reductive elimination for C-C bond formation only gave moderate yield (40%) of **3a** (Entry 8). Using a stronger base (Cs_2CO_3) or in the absence of additives gave no product (Entries 9-10). The solvent system of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1) was found to give the best yields while using other solvents such as dichloroethane (DCE), toluene, and tetrahydrofuran (THF) gave no product even at a higher reaction temperature (60 °C) (Entries 11-13).

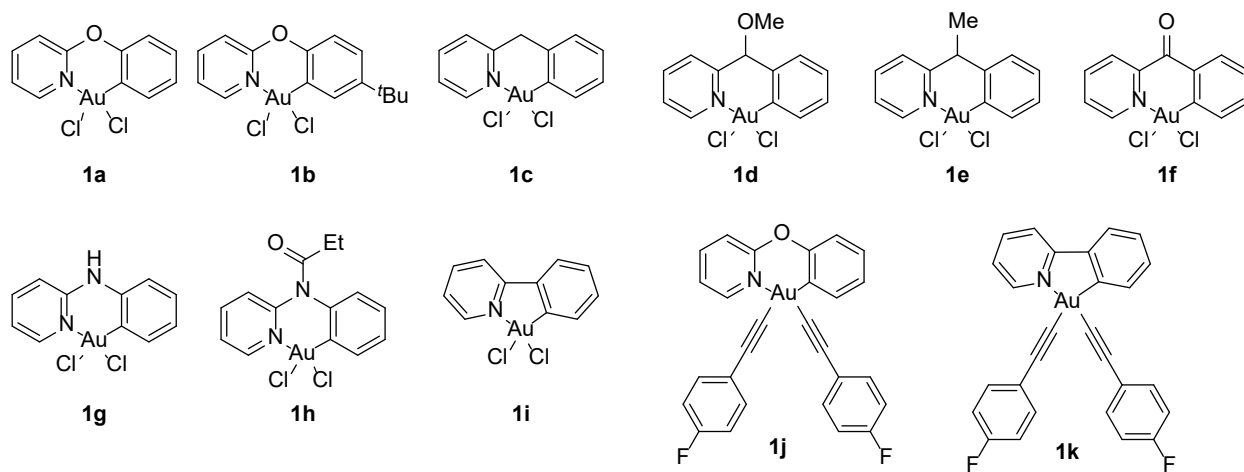


Figure 1. Structures of cyclometalated gold(III) complexes $[Au(C^N)L_2]$ **1a-1i** (L = chloride) and **1j-1k** (L = 4-fluorophenylacetylene).

Table 1. Optimization of Reaction Conditions^a

Entry	2a (equiv.)	Additive	Solvent	Temp. (°C)	Yield (%)
1	1	NaHCO ₃	CH ₃ CN/H ₂ O (1:1)	40	40
2	2	NaHCO ₃	CH ₃ CN/H ₂ O (1:1)	40	48
3	5	NaHCO ₃	CH ₃ CN/H ₂ O (1:1)	40	74
4	5	NaHCO ₃	CH ₃ CN	40	60
5	5	NaHCO ₃	H ₂ O	40	68
6	5	K ₂ CO ₃	CH ₃ CN/H ₂ O (1:1)	40	82
7	5	Et ₃ N	CH ₃ CN/H ₂ O (1:1)	40	66
8	5	PPh ₃	CH ₃ CN/H ₂ O (1:1)	40	40
9	5	Cs ₂ CO ₃	CH ₃ CN/H ₂ O (1:1)	40	-
10	5	-	CH ₃ CN/H ₂ O (1:1)	40	-
11	5	K ₂ CO ₃	DCE	60	-
12	5	K ₂ CO ₃	Toluene	60	-
13	5	K ₂ CO ₃	THF	60	-

^a The reactions were carried out with [Au(pop)Cl₂] **1a** (0.02 mmol, 1 equiv.), phenylacetylene **2a** and additive (5 equiv.) in 4 mL of solvent for 16 h.

Expansion of the substrate scope of this reaction using a series of 6-membered ring [Au(C[^]N)Cl₂] **1a-1i** with different groups incorporated between their aryl and pyridine rings gave alkylation products **3a-3h** in 48-74% yields (Figure 2). Electron donating (-OMe and -Me) or withdrawing (-

F) groups on the *para* position of the phenylacetylene would not affect the alkynylation of 2-phenoxy pyridine (Hpop) significantly to afford **3i-3k** with up to 64% yields. Furthermore, the alkynylation of 2-phenoxy pyridine (Hpop) with terminal alkynes bearing aliphatic or heterocyclic groups afforded the alkynylation products **3l-3o** in 28-65% yields. A model reaction of the alkyne-linked peptide using amide-linked aliphatic alkyne **2i** also proceeded smoothly to afford the alkynylation product **3p** in 76% isolated yields. To our delight, the chemical structure of the product **3f-1** obtained by reacting 6-membered ring complex [Au(pcp)Cl₂] (Hpcp = 2-benzoylpyridine) **1f** with 4-fluorophenylacetylene **2d** was supported by X-ray crystallography (Figure S20 and Table S2). These findings indicated the alkynylation has high functional group compatibility with the gold(III) metallocycles and terminal alkynes.

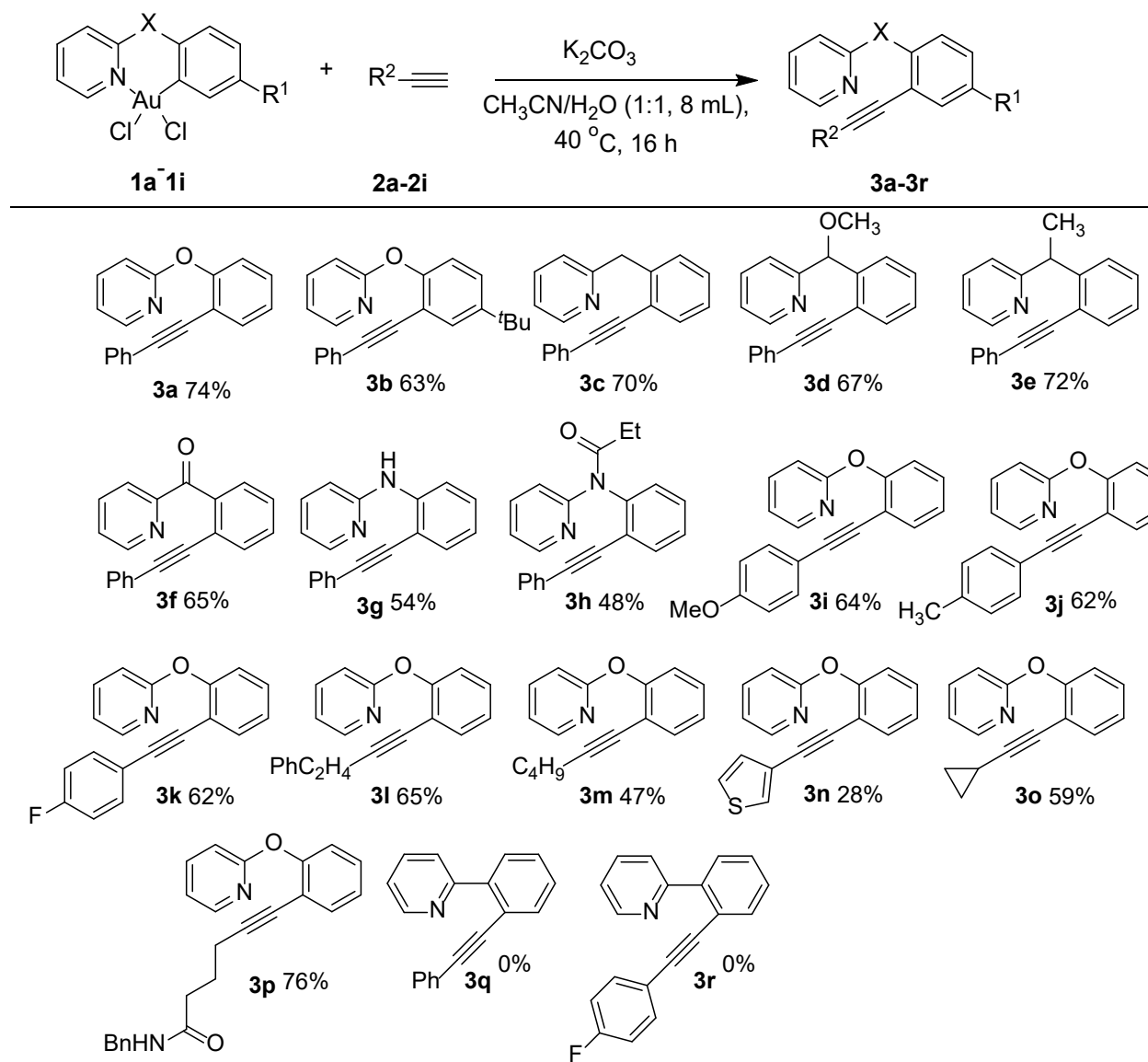


Figure 2. Expansion of the substrate scope. The reactions were carried out with cyclometalated gold(III) complexes **1a-1i** (0.1 mmol), terminal alkynes **2a-2i** (0.5 mmol), and K_2CO_3 (0.5 mmol) in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, 8 mL) at 40 °C for 16 h.

Interestingly, when alkynes were treated with 5-membered ring cyclometalated gold(III) complex $[\text{Au}(\text{ppy})\text{Cl}_2]$ **1i** (Hppy = 2-phenylpyridine), no alkylation product **3q** or **3r** was observed (Figure 2), which was consistent with our previous observation in C-S bond formation

reaction. The difficulty to undergo reductive elimination of **1i** with terminal alkynes was also pointed out by Shi *et al.*^{21a} To provide insight on alkylation of 2-arylpyridines from cyclometalated gold(III) complexes and terminal alkynes, we attempted to identify the plausible reaction intermediates by ¹⁹F-NMR analysis and therefore dialkynyl gold(III) complexes **1j** and **1k** were prepared (Figure 3). Dialkynyl gold(III) complex **1j** (0.01 mmol) was stirred in CD₃CN (0.6 mL) at 40 °C for 3 h. The crude reaction mixture was subjected to ¹⁹F NMR analysis using fluorobenzene (-114.93 ppm) as reference. ¹⁹F-NMR analysis indicated that **1j** (-114.17 and -114.73 ppm) underwent reductive elimination to form the alkylation product **3k** (-112.21 ppm) in 83% yield and diyne **2d'** (-109.98 ppm) (Figure S1), while no reductive elimination product was observed in the reaction of 5-membered ring gold(III) complex [Au(pop)Cl₂] **1i** with **2d** (Figure S2), indicating the reductive elimination of gold(III) complex was significantly affected by the ring size of the gold(III) metallocycles.

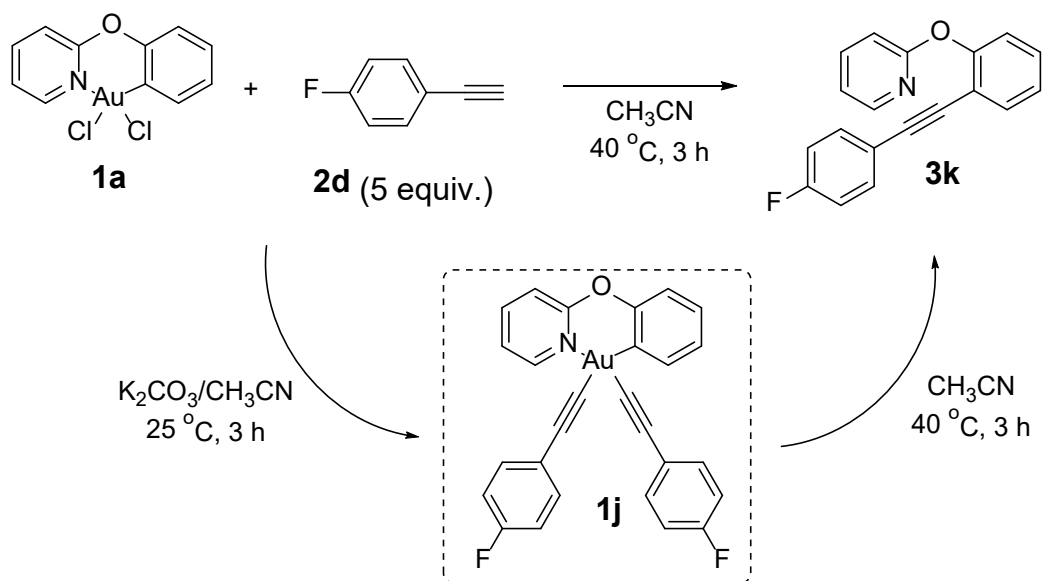
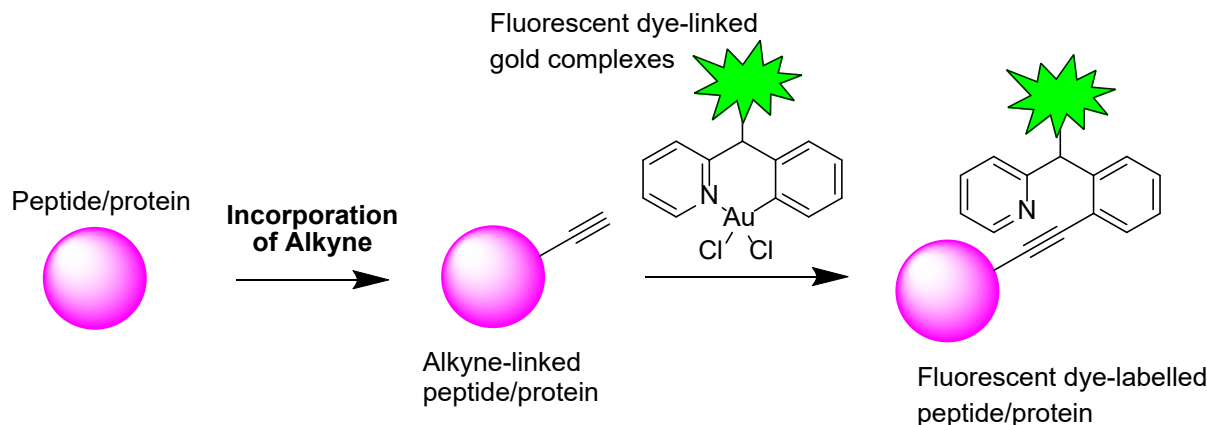


Figure 3. Proposed reaction pathway of alkylation between [Au(pop)Cl₂] **1a** and 4-fluorophenylacetylene **2d** via dialkynyl gold(III) complex **1j** as the reaction intermediate.

With the promising findings of the alkylation with 6-membered ring cyclometallated gold(III) complexes $[\text{Au}(\text{C}^{\wedge}\text{N})\text{Cl}_2]$ under mild reaction conditions, we envision that the present reaction would be further developed into a new method for selective modification of alkyne-linked peptides and proteins (Scheme 2).



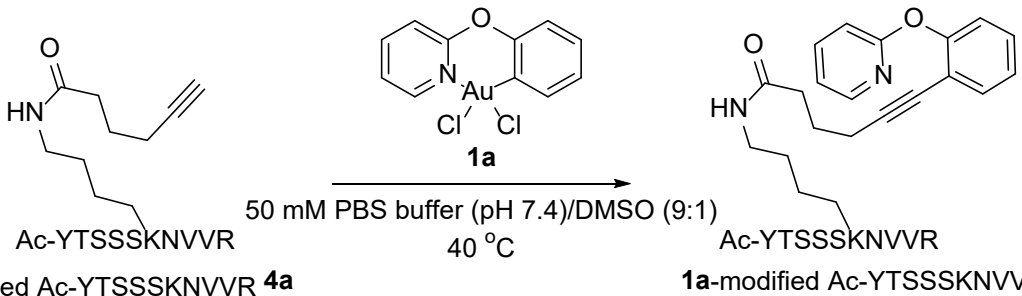
Scheme 2. Chemoselective modification of alkyne-linked peptides/proteins.

As a proof-of-concept, the alkyne handle was first incorporated into a model peptide, Ac-YTSSSKNVVR **4b**, with 5-hexynoic acid *N*-hydroxysuccinimide ester **2j** to obtain alkyne-linked Ac-YTSSSKNVVR **4a** in 97% conversions (Figures S3-S5). The reaction conditions were then screened by treatment of alkyne-linked Ac-YTSSSKNVVR **4a** (0.05 mM) with 5 equivalents of $[\text{Au}(\text{pop})\text{Cl}_2]$ **1a** (Hpop = 2-phenoxy pyridine) in 50 mM PBS buffer (pH 7.4)/DMSO (9:1) at 40 °C for 2 h and 4 h and afforded **1a**-modified Ac-YTSSSKNVVR (Figure 4a) in 59% and >99% conversions, respectively (Table 2, entries 1 and 2). LC-MS/MS analysis indicated that the modification was on the alkyne-linked lysine residue, while other residues of the peptide remained intact (Figure 4b). Treatment of Ac-YTSSSKNVVR **4b** with $[\text{Au}(\text{pop})\text{Cl}_2]$ **1a** under the same

conditions resulted in no conversion of the peptide, indicating that the modification was selective towards the alkyne moiety (Entry 3).

Peptide modification using 6-membered ring cyclometalated gold(III) complex [Au(pcp)Cl₂] **1f** (Hpcp = 2-benzoylpyridine) also proceeded smoothly, giving overall conversions of peptide **4a** in > 99% conversions with 20% conversions as **1f**-modified Ac-YTSSSKNVVR and 79% conversions as hydrated **1f**-modified Ac-YTSSSKNVVR (Entry 4; Figures S9-S12). Notably, consistent with the previous observation, the ring sizes of the gold(III) metallocycles (6- vs 5-membered ring) also affected the peptide modification. No desired modified product observed by treatment of 5-membered ring [Au(ppy)Cl₂] **1i** (Hppy = 2-phenylpyridine) with alkyne-linked Ac-YTSSSKNVVR **4a** (Entry 5), suggesting that 6-membered ring cyclometalated gold(III) complexes were much more efficient than the 5-membered ring analogues in peptide modification.

Besides, we conducted time course experiments to test the effect of pH values on the modification. By changing the buffer from slightly acidic to basic, the conversions enhanced from 64% to > 99% within 4 h, which was consistent with the previous findings that basic reaction conditions could facilitate the alkynylation reaction (Figure 5).

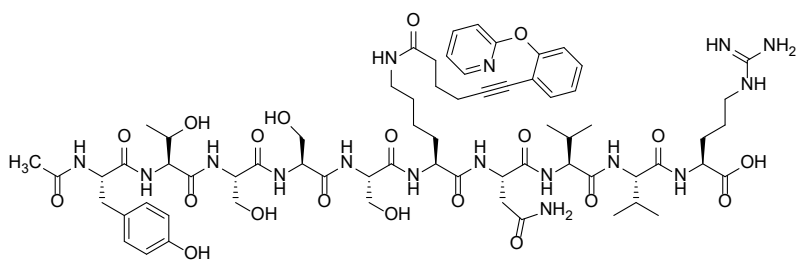
Table 2. Condition Screening of Peptide Modification^a

Reaction scheme showing the modification of alkyne-linked peptide **4a** (Ac-YTSSSKNVVR) with gold(III) complex **1a** (Au(C^N)Cl₂) in 50 mM PBS buffer (pH 7.4)/DMSO (9:1) at 40 °C, yielding **1a**-modified Ac-YTSSSKNVVR.

Entry	Peptide	[Au(C ^N)Cl ₂]	Conversion (%)
1	4a	1a	59 ^b
2	4a	1a	> 99
3	4b ^c	1a	0
4	4a	1f	> 99 ^d
5	4a	1i	0

^a Reactions were carried out with 0.05 mM of peptide (1 equiv.) with gold(III) complex (5 equiv.) in 50 mM PBS buffer (pH 7.4)/DMSO (9:1) at 40 °C for 4 h. ^b Reaction was carried out for 2 h. ^c Peptide sequence of **4b** is Ac-YTSSSKNVVR. ^d With **1f**-modified Ac-YTSSSKNVVR in 20% conversions and hydrated **1f**-modified Ac-YTSSSKNVVR in 79% conversions.

(a)



(b)

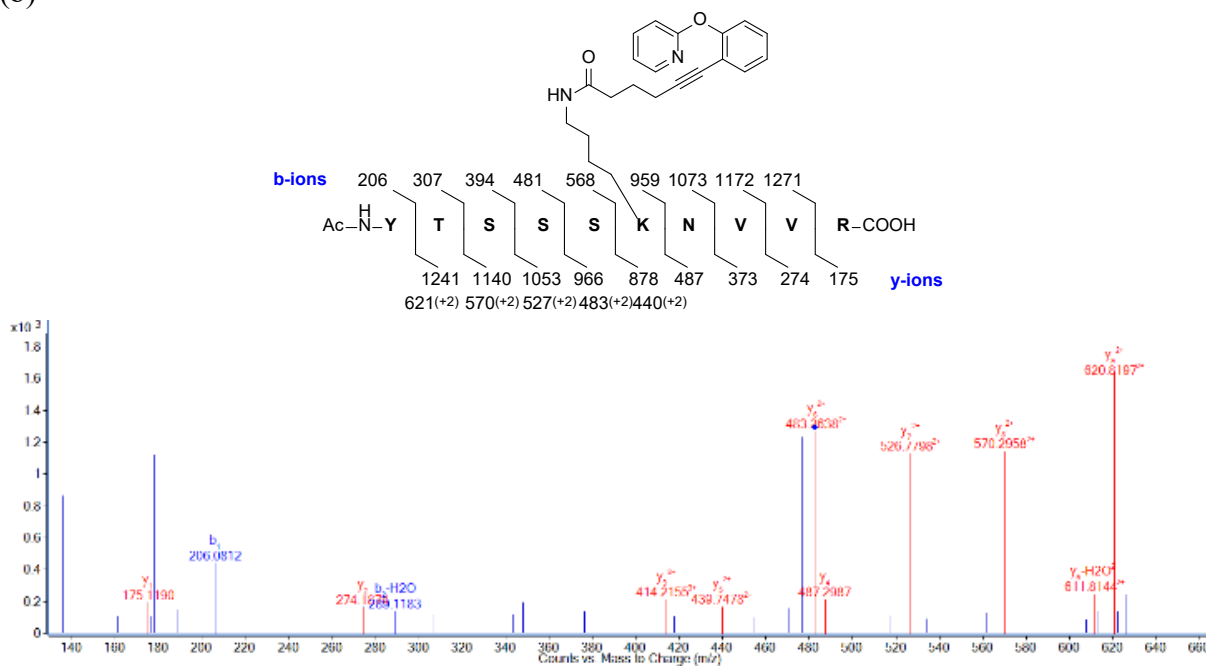


Figure 4. (a) Structure of **1a**-modified Ac-YTSSSKNVVR. (b) MS/MS spectrum of **1a**-modified Ac-YTSSSKNVVR.

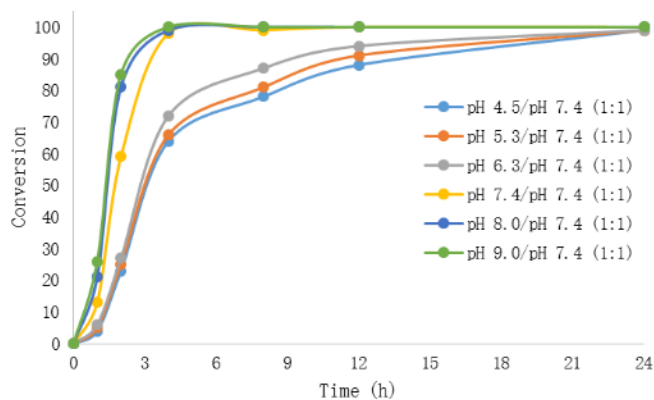
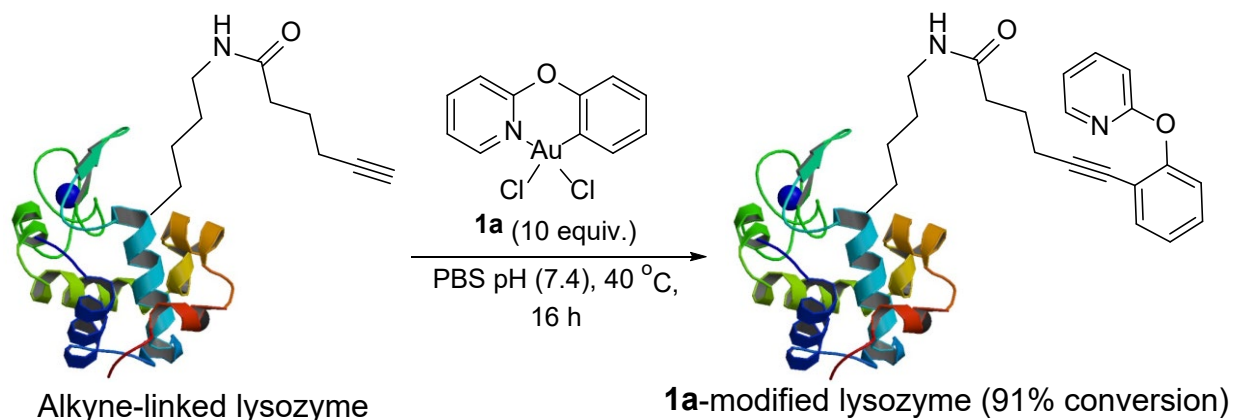


Figure 5. Time course of the **alkynylation** of alkyne-linked Ac-YTSSSKNVVR **4a** with cyclometalated gold(III) complex [Au(pop)Cl₂] **1a** in 50 mM PBS buffer at different pH values.

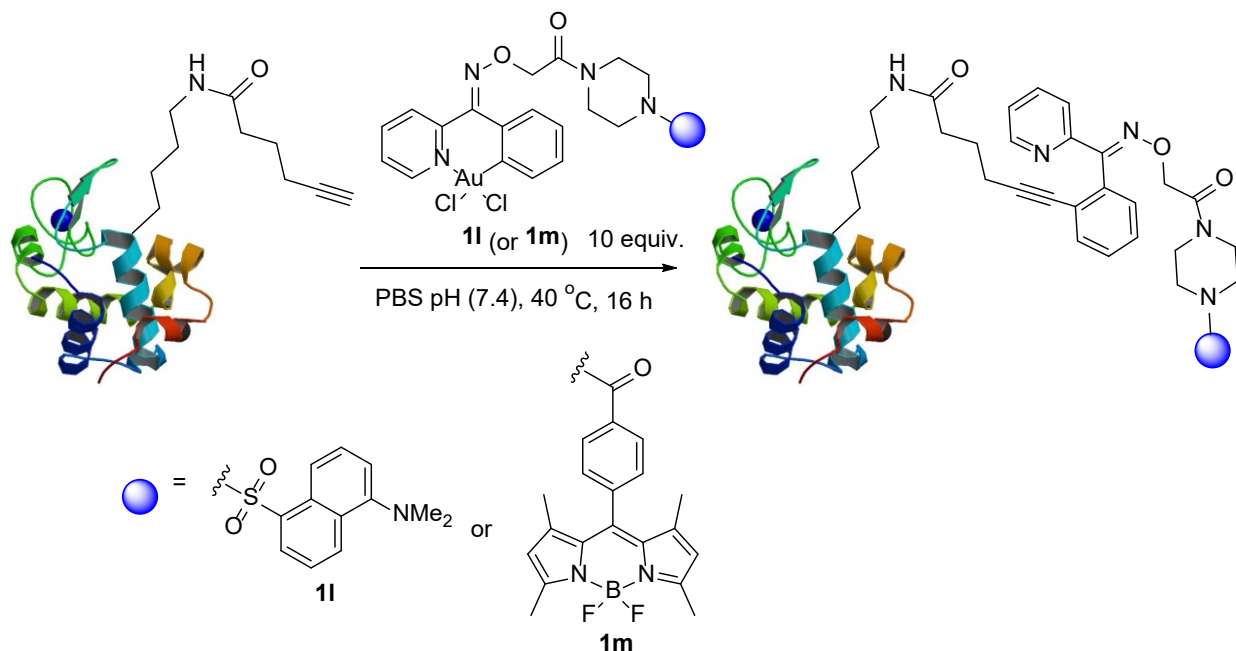
We have reported that cysteine residues in peptides and proteins can undergo reductive elimination with cyclometalated gold(III) complexes $[\text{Au}(\text{C}^{\wedge}\text{N})\text{msen}]$,¹⁸ however, the intrinsically sparse occurrence (1.7%) of cysteine in proteins may limit the application of this chemoselective cysteine modification for biological studies.^{1a,2} Therefore, we sought to use these newly developed cyclometalated gold(III) complexes $[\text{Au}(\text{C}^{\wedge}\text{N})\text{Cl}_2]$ as efficient chemoselective reagents to modify alkyne-linked proteins. With lysozyme (PDB ID: 1DPX) chosen as a protein example, the terminal alkyne was incorporated to the lysine residues on the lysozyme by reaction with 5-hexynoic acid *N*-hydroxysuccinimide ester **2j** (Figure S13). After removing the excess unreacted **2j** in the reaction mixtures by centrifugal filtration, the alkyne-linked lysozyme was then reacted with 10 equivalents of $[\text{Au}(\text{pop})\text{Cl}_2]$ **1a** in 50 mM PBS buffer (pH 7.4) at 40 °C for 16 h (Scheme 3). The product formation was confirmed by LC-MS/MS analysis of the trypsin digested peptide sequences (Figures S14-S16).



Scheme 3. Modification of alkyne-linked lysozyme.

Labelling of proteins with fluorescent tags provides an opportunity for convenient protein staining in SDS-PAGE gel using fluorescent analysis and also has potential for *in vivo* tracking the

uptake and physiological parameters.^{1c} As the alkylation reaction could be successfully applied on protein modification, we next demonstrated the utility of this reaction for fluorescent labelling of proteins. Cyclometalated gold(III) complexes **1l** and **1m** linked with dansyl and BODIPY groups, respectively, were synthesized for fluorescent labelling of alkyne-linked lysozyme by alkylation reaction (Scheme 4), and the fluorescent labelling were observed by SDS-PAGE analysis (Figure S17) and LC-MS (Figure S18-S19). According to the results of SDS-PAGE analysis, dansyl (**1l**)-modified lysozyme and BODIPY (**1m**)-modified lysozyme were observed using fluorescent analysis while no fluorescent signal was observed for **1a**-modified lysozyme. Employing Coomassie blue staining on the same gel, deep blue colour signals of **1a**, dansyl (**1l**) and BODIPY (**1m**)-modified proteins could be observed, indicating that fluorescent tags have been labelled on the proteins using the alkylation approach.



Scheme 4. Fluorescent labelling of alkyne-linked lysozyme.

In 2018, Tanaka *et al.* discovered that gold catalysts containing 2-benzoylpyridine ligand (Hpcp) exhibited significantly high reactivity in protein labelling. It was suggested that the cyclometalated gold(III) complex with 2-benzoylpyridine ligand and propargyl esters underwent a novel C(sp²)-C(sp) aryl-alkynyl cross-coupling through reductive elimination.^{20b} The resulting activated ester intermediate is of great importance in the future development of gold-based *in vivo* bioconjugation applications. In our present work, we conducted more detailed investigations on the alkylation reaction of 6-membered ring cyclometalated gold(III) complexes [Au(C[^]N)Cl₂] with various terminal alkynes including alkyne-linked peptides and proteins. Thus, our present work has provided more experimental findings to support the discovery made by Tanaka.

Conclusion

In summary, we have developed a new alkylation reaction of 6-membered ring cyclometalated gold(III) complexes [Au(C[^]N)Cl₂] with terminal alkynes under mild conditions. We have applied the alkylation reaction for selective modification of alkyne-linked peptides and proteins. Using lysozyme as an protein example, the chemoselectivity of this reaction has been demonstrated by labelling fluorescent tags on alkyne-linked lysozyme by using fluorescent dye-linked cyclometalated gold(III) complexes.

Data availability

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

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, characterizations, NMR spectra, LC-MS and LC-MS/MS analysis of the products (PDF)

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TOC

