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2-Benzoylpyridine ligand complexation with gold critical for propargyl ester-based protein labeling

Yixuan Lin[+],[a,b] Kenward Vong[+],[a] Koji Matsuoka,[b] and Katsunori Tanaka*[a,c,d]

Abstract: Previously, Au(III) complexes coordinated with 2-benzoylpyridine ligand, BPy-Au, were prebound to a protein and used to discover a novel protein-directed labelling approach with propargyl ester functional groups. In this work, further examination discovered that gold catalysts devoid of the 2-benzoylpyridine ligand (ex/ NaAuCl4) had significantly reduced levels of protein labeling. Mechanistic investigations then revealed that BPy-Au and propargyl esters undergo a rare example of C(sp^2)-C(sp) aryl-alkynyl cross coupling, likely via spontaneous reductive elimination. Overall, these observations appear to suggest that BPy-Au-mediated, propargyl ester-based protein labeling acts through an activated ester intermediate, which contributes to our understanding of this process and will aid the expansion/optimization of gold-catalyst usage in future bioconjugation applications, especially in vivo.

Protein bioconjugation, described as the technique of covalently linking biological molecules of interest to proteins, has been the focus of numerous studies in the field of bioorganic chemistry.[1] Moving away from traditional bioconjugation approaches, such as lysine-selective (ex/ NHS ester acylation,[2] 6π-azaelectrocylization of imines,[3] etc) and cysteine-selective methods,[4] there has been a growing interest towards developing methods of metal-catalyzed protein bioconjugation. Several working metal-catalyzed approaches for both natural and unnatural amino acids have already been shown in literature,[1h-k] where they have largely succeeded in overcoming the challenges of using metals under biological environments (ex/ maintaining reactivity in the presence of oxygen, water, and biological thiols). Of particular interest to this paper is protein bioconjugation based on Au(III) metal catalysts; notable examples being cysteine conjugation via allene-based probes to generate 1,2/1,3-adducts,[5] and cysteine conjugation to the ligands of cyclometallated Au complexes via reductive elimination.[6] However, these methods generally have limited use for proteins with low levels of surface accessible free cysteines (ex/ albumin).

One intriguing example of gold catalyzed protein bioconjugation was recently described by our group.[7] In this study, the novel concept of organ-targeting, metal-carrier glycoalbumins was developed (Figure 1). First, to impart organ-targeting properties, glycoalbumins (glycan-linked albumins) were synthesized according to previous studies that discovered specific glycan-linkages could influence biodistribution in mice through the multivalency effects of glycoclusters.[8] As summarized in Figure 1A, homogenous clusters of α(2,3)-disialo-, α(2,6)-disialo-, and galactosyl-linked glycoalbumins showed preferential accumulation onto tumor tissues, the liver, and intestines, respectively. Next, by using the natural ligand binding affinities of human serum albumin, these glycoalbumins could be further modified to become transition metal carriers. This was done by linking desired metal complexes to a coumarin moiety, which is known as a high affinity binder to the IB subdomain of albumin.[9] In this manner, a 2-benzoylpyridine-Au(III) complex (BPy-Au) was anchored to albumin, which led to the serendipitous discovery of a protein-directed labeling approach with propargyl esters via gold catalysis (Figure 1B). Using this system, the first example of localized protein labeling on the surface of targeted organs *in vivo* was successfully shown within live mice.[7]

Herein, this paper describes our initial investigations to gain a further understanding of the protein labeling mechanism mediated by BPy-Au complexes. As expected, our results show that unbound, free-in-solution BPy-Au also facilitates protein labeling (Figure 1C). However, we were surprised to find that with other Au(III) catalysts lacking the 2-benzoylpyridine ligand, there was a significant decrease in protein labeling (Figure 1D). Coupled with preliminary mechanistic investigations, this work aims to communicate the novel and unexpected reactivity of 2-benzoylpyridine-Au(III) complexes, and its importance to our previously developed approach of organ-targeting, metal-carrier glycoalbumins.[7]

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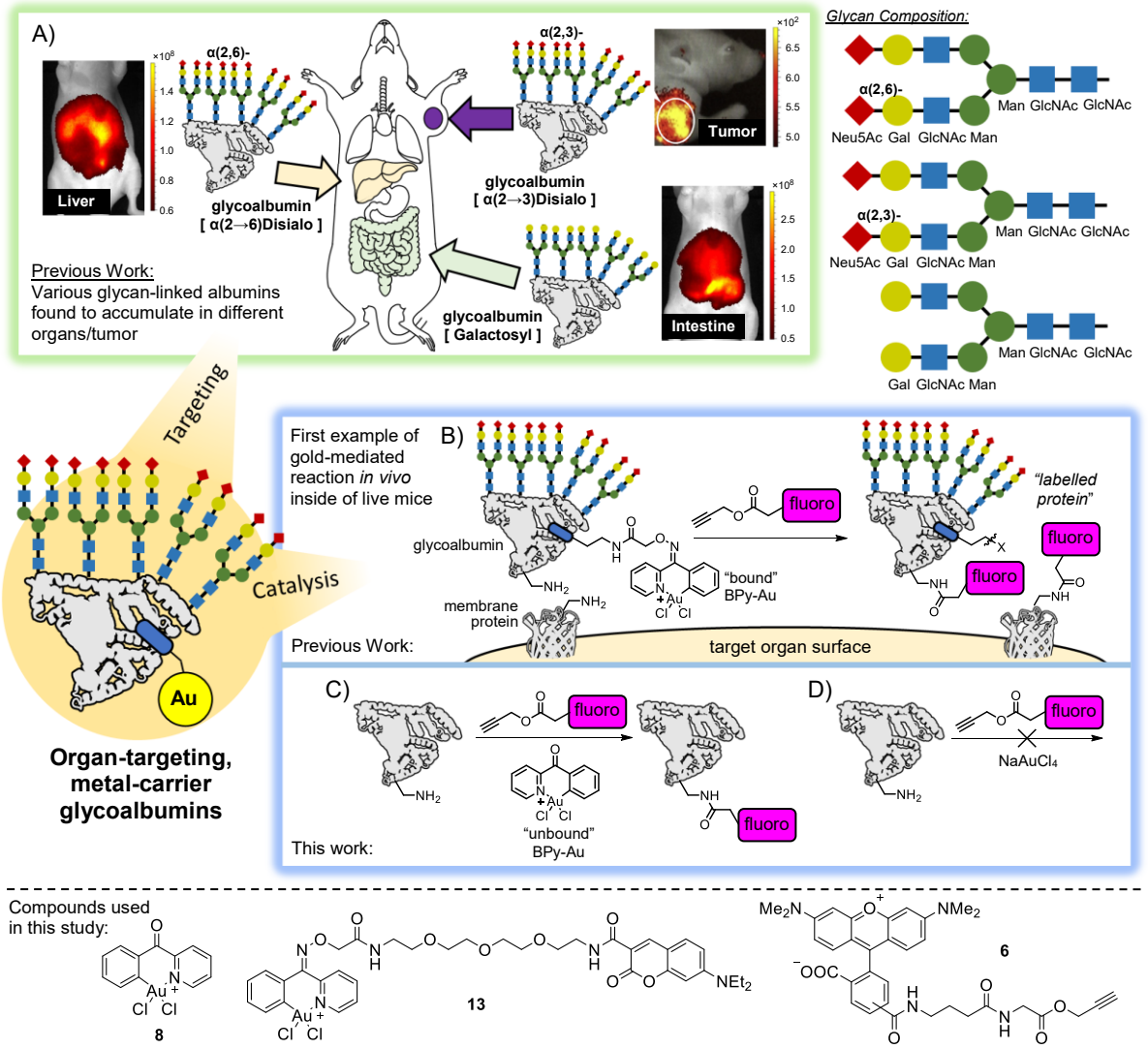

Figure 1. Overview of organ-targeting, metal-carrier glycoalbumins. A) Organ-targeting properties are conferred via albumin linkage to various types of glycans. The composition of α(2,3)-disialo-, α(2,6)-disialo-, and galactosyl-based glycans are as shown. B) Metal-carrier properties are given by anchoring a BPy-Au complex via coumarin linkage (compound **13**). Localized protein labeling on targeted mouse organs was observed. Further investigations into reactivity show that C) unbound BPy-Au **8** also facilitates propargyl ester-based protein labeling and D) other gold catalysts devoid of 2-benzoylpyridine complexation have significantly reduced levels of protein labeling.

To begin, various compounds were synthesized, as outlined in the Supplementary Information. The propargyl ester-containing fluorophore **6** was synthesized in 32% overall yield in accordance to previous studies.[7, 10] In terms of gold catalysts, BPy-Au **8** was synthesized according to literature.[6, 11] By further adding a coumarin moiety to **8** via a PEG linker, the synthesis of coumarin-BPy-Au **13** was obtained in 7% overall yield.[7] For $NaAuCl_4$, a commercial source was used. For the construction of albumin complexed with coumarin-BPy-Au **13**,[7] the procedure outlined in the Supplementary Information was followed. Protein complex formation was further confirmed via binding fluorescence measurements, as explained in Figure S2.

In this study, the degree of labeling reactivity between "unbound gold" and "bound gold" protein was compared. As depicted in Figure 2A, “unbound gold” represents a situation where albumin labeling is facilitated by free-in-solution BPy-Au **8**. On the other hand, "bound gold" represents when the gold catalyst is inherently anchored to albumin, which is done through the use of coumarin-BPy-Au **13**. As a negative control, metal-free “no gold” conditions were also tested.

Upon incubation with TAMRA-linked propargyl ester **6**, time-dependent fluorescence measurements of the three reaction conditions were taken. As shown in Figure 2B and S3, observations indicate that protein labeling with free, unbound gold catalyst **8** (Figure 2B, black diamond) proceeded to a greater degree compared to protein bound gold catalyst (Figure 2B, grey squares).

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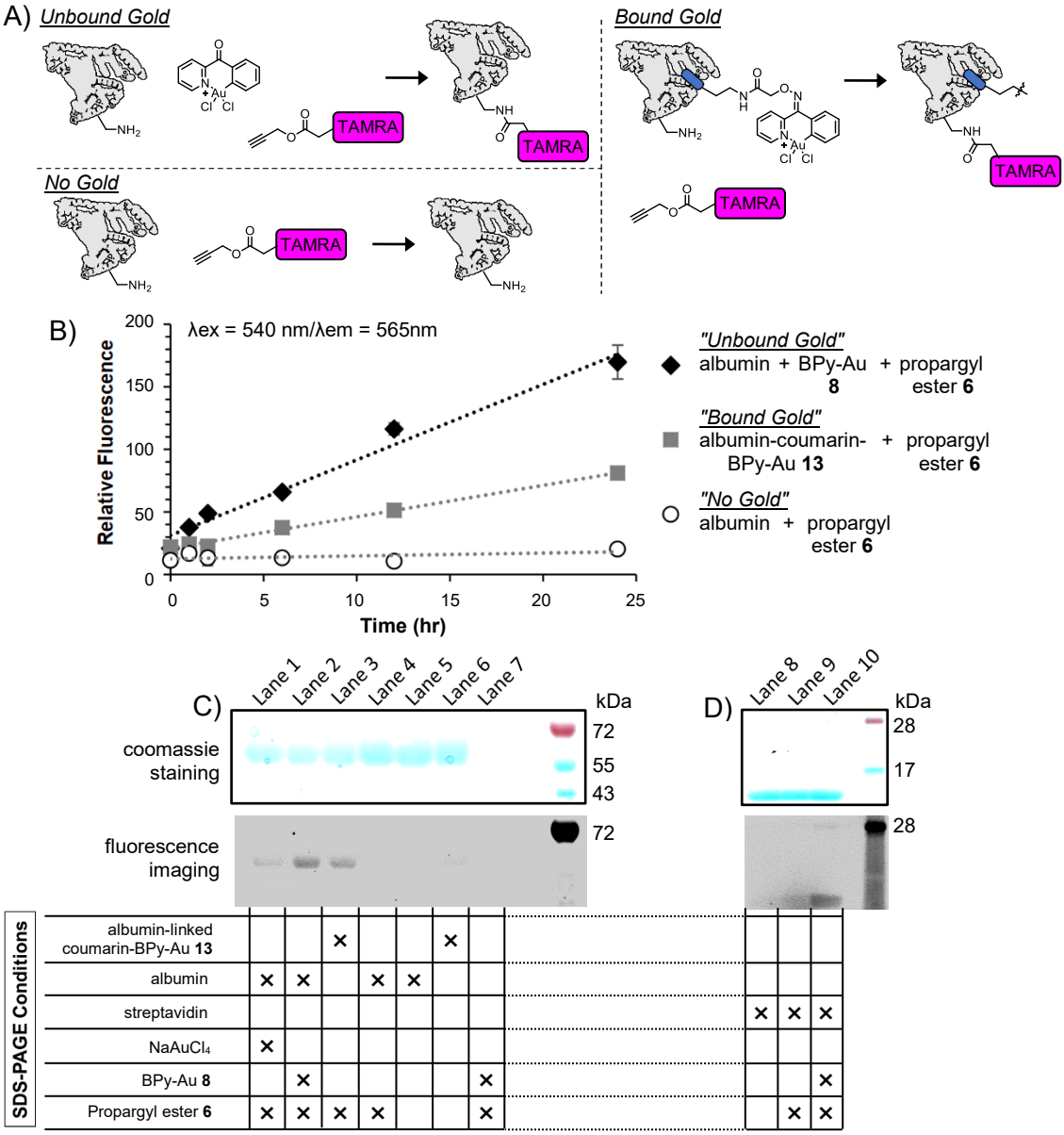

Figure 2. Protein labeling experiments to confirm reactivity. A) Illustrative representation of unbound, bound, and no gold protein labeling conditions. B) Time-dependent monitoring of protein fluorescence under unbound, bound, and no gold protein labeling conditions. C) SDS-PAGE analysis to determine propargyl ester-based labeling of albumin under various conditions. D) SDS-PAGE analysis to determine propargyl ester-based labeling of streptavidin under various conditions.

To further confirm albumin labeling, various reaction conditions were analyzed by SDS-PAGE (Figure 2C and S4). Results show that albumin labeled under "unbound gold" conditions (Figure 2C, Lane 2) was more fluorescent compared to albumin labeled under "gold bound" conditions (Figure 2C, Lane 3); densitometry analysis of these bands are shown in the Supporting Information. In terms of the control conditions, none of albumin incubated with propargyl ester **6** (Figure 2C, Lane 4), albumin only (Figure 2C, Lane 5), albumin-linked coumarin-BPy-Au only (Figure 2C, Lane 6), and reagents **8** and **6** only (Figure 2C, Lane 7) showed fluorescent albumin bands.

During the course of this study, an unexpected observation noticed that propargyl ester-based protein labeling proceeded significantly more with Au(III) catalysts coordinated to 2-benzoylpyridine ligands (ex/ BPy-Au **8**, coumarin-BPy-Au **13**) compared to others (ex/ $NaAuCl_4$). This reactivity was confirmed by SDS-PAGE analysis, where albumin labeled under $NaAuCl_4$ incubation (Figure 2C, Lane 1) was shown to emit significantly lower levels of fluorescence compared to albumin labeled under BPy-Au containing conditions.

Another experimental condition confirmed that BPy-Au-catalyzed, propargyl ester-based labeling could be transferred to other proteins. With streptavidin, various conditions were tested and analyzed by SDS-PAGE (Figure 2D). Using BPy-Au **8** as a catalyst, labeling of streptavidin was successfully observed (Figure 2D, Lane 10) and was more fluorescent compared to the controls; streptavidin incubated with propargyl ester **6** (Figure 2D, Lane 9), and streptavidin only (Figure 2D, Lane 8).

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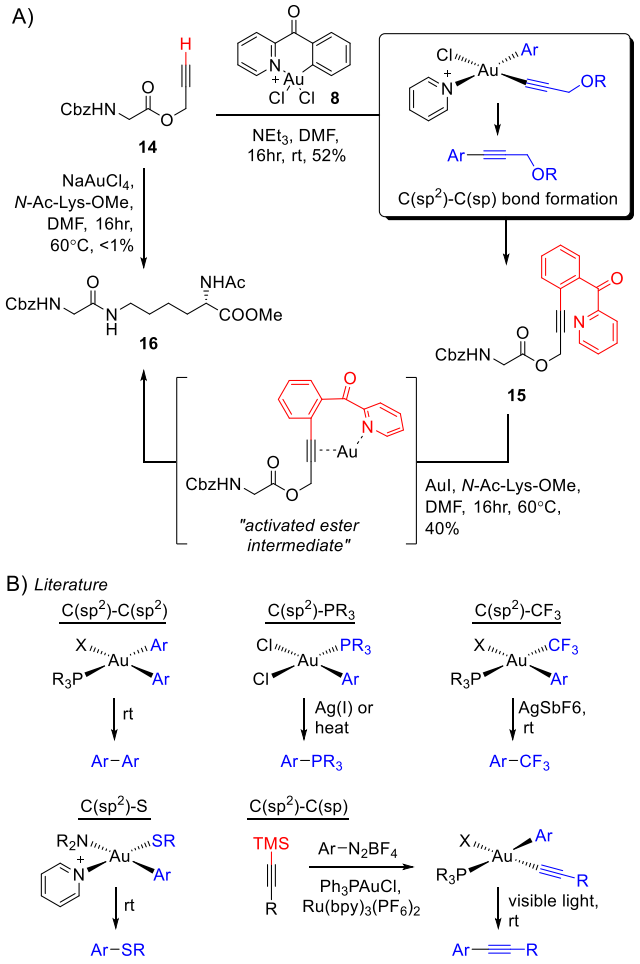

Figure 3. A) Observed cross-coupling reaction between propargyl ester **14** and BPy-Au **8** to produce the active ester intermediate **15**, which forms through reductive elimination of the formed Au(III) complex to trigger C(sp^2)-C(sp) bond formation. Subsequent studies to monitor lysine amidation reveal higher yields are obtained with activated ester **15** compared to ester **14**. B) Select literature examples of other types of bond formation via reductive elimination of Au(III) complexes under mild conditions.

To further understand the importance and role of 2-benzoylpyridine ligand coordination for gold-catalyzed propargyl ester protein ligation, mechanistic investigations were conducted. Preliminary results show that when propargyl ester compound **14** was incubated overnight in DMF under basic conditions with only BPy-Au **8**, the unexpected product **15** could be cleanly purified in 52% yield (Figure 3A). Other constituents in the reaction mixture were found to be unreacted starting material. In principal, compound **15** is the cross-coupling product between the propargyl ester and 2-benzoylpyridine. It is hypothesized that this transformation likely creates an “activated ester intermediate” when coordinated with remaining gold species in solution. This could then lead to an increase in the rate of amide bond formation with protein surface amines (ex/ lysines). As a model study, compound **15** was incubated with AuI, which acts as a representative gold(I) species that would be present following reductive elimination-based cross coupling. Further reaction with *N*-Ac-Lys-OMe then gave the expected amide product **16** in 40% yield. This is in contrast to the use of propargyl ester **14** under similar conditions, which results in almost negligible yield.

Additionally, it should be noted that stable Au(III) complexes have shown the inherent ability in several literature examples to undergo spontaneous reductive elimination to form C(sp^2)-C(sp^2),[12] C(sp^2)-S,[6] C(sp^2)-P,[13] and C(sp^2)-CF_3 bonds,[14] all which proceed under various mild conditions (Figure 3B). To the best of our knowledge, our example is the first to facilitate C(sp^2)-C(sp) bond formation from terminal alkynes. The only other relevant example of C(sp^2)-C(sp) bond formation requires visible light mediation and the use of alkynyltrimethylsilanes (transmetallation from the organosilane is said to be needed for reactivity).[15]

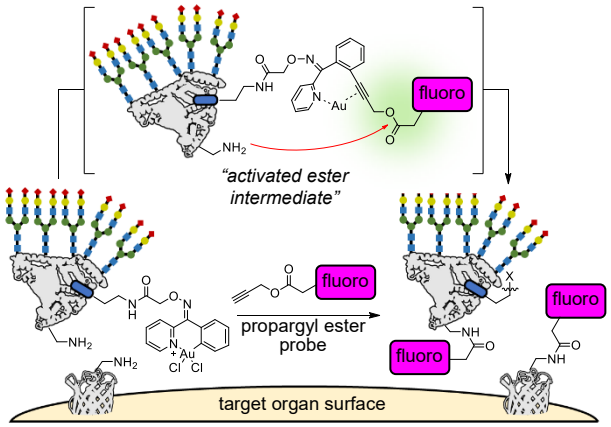

Figure 4. Proposed mechanism of BPy-Au-mediated, propargyl ester-based protein labeling, which likely acts through ester activation via initial aryl-alkynyl cross coupling.

In conclusion, this work was able to gather sufficient evidence to formulate two new conclusions regarding BPy-Au-mediated, propargyl ester-based protein labeling. First, protein labeling does not necessitate the gold catalyst to be prebound to a protein, which was the situation mainly employed in a previous work by our group.[7] This study shows that unbound, free-in-solution gold catalysts also have the ability to facilitate propargyl ester-based protein labeling, which consistently shows a higher degree of reactivity compared to its "bound gold" equivalent. The differences in labeling reactivities is likely explained by the fact that "bound gold" reactivity is limited to nearby surface amine residues, whereas "unbound gold" reactivity possibly acts on all protein surface amines. In addition, protein labeling is not limited to just albumin, as reactivity with streptavidin was also observed.

Second, an unexpected observation showed that if gold catalysts are devoid of 2-benzoylpyridine ligand coordination (ex/ $NaAuCl_4$), protein labeling proceeded to a significantly lower degree. This suggests that BPy-Au-mediated protein ligation likely acts through an activated ester intermediate, which then increases the rate of amide bond formation with protein surface amines, as depicted in Figure 4. In addition, the observation of the unexpected activated ester intermediate formation by aryl-alkynyl cross coupling represents a rare synthetic example of C(sp^2)-C(sp) bond formation from the spontaneous reductive elimination of Au(III) complexes.

It should be noted that gold itself is known to be a π-philic metal catalyst. Although the use of BPy-Au catalyst complexes

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results in significantly higher amidation yields, we do see trace amounts of desired product using unligated gold, such as $NaAuCl_4$ (Figure 2C-Lane 1 shows faint protein conjugation; direct conversion of **14** to **16** gives trace yields <1%). In this case, we believe unligated gold may weakly activate the propargyl ester by coordinating to the alkyne moiety. One could surmise that a low level of propargyl ester based-conjugation on glycoalbumin surfaces may in fact be facilitated by simple gold-coordination from the BPy-Au moiety. This would have to take place catalytically before the spontaneous reductive elimination of the Au(III) complex occurs, which we have found takes a considerable amount of time (overnight reaction).

Overall, significant advances to the understanding of BPy-Au mediated propargyl ester protein ligation were made. Coupled with the continued development of organ-targeting, metal-carrier glycoalbumins, this work will undeniably aid the development of future therapeutic applications, some of which focus on anticancer approaches that could use gold-mediated labeling of biomarkers (ex/ antigens) to targeted tissue surface proteins (ex/ tumors).

Acknowledgements

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Keywords: Gold catalysis • protein conjugation • aryl-alkynyl cross coupling

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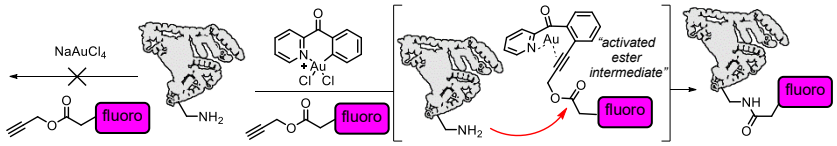

2-Benzoylpyridine-Au(III) complexes mediate propargyl ester protein labeling likely through an activated ester intermediate. Previous research showed gold-catalyzed protein bioconjugation could proceed *in vivo*, but it's mechanism was not fully understood. In this work, further examination discovers that coordinated 2-benzoylpyridine ligands are crucial for reactivity. This is hypothesized to proceed through a rare example of $C(sp^2)$-C(sp) aryl-alkynyl cross coupling.

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