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To be cited as: ChemBioChem 10.1002/cbic.201600578

Link to VoR: https://doi.org/10.1002/cbic.201600578

A bifunctional amino acid enables both covalent chemical capture and isolation of *in vivo* protein-protein interactions

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Abstract: *In vivo* covalent chemical capture via photo-activatable unnatural amino acids (UAAs) is a powerful tool for the identification of transient protein-protein interactions (PPIs) in their native environment. However, the isolation and characterization of the crosslinked complexes can be challenging. Here, we report the first *in vivo* incorporation of the bifunctional UAA BPKyne for the capture and direct labeling of crosslinked protein complexes through post-crosslinking functionalization of a bioorthogonal alkyne handle. Using the prototypical yeast transcriptional activator Gal4 we demonstrate that BPKyne is incorporated at the same level as the commonly used photo-activatable UAA pBpa and effectively captures the Gal4-Gal80 transcriptional complex. Post-crosslinking, the Gal4-Gal80 adduct was directly labeled by reaction of the alkyne handle with a biotin-azide probe, which enabled facile isolation and visualization of the crosslinked adduct from whole cell lysate. This bifunctional amino acid extends the utility of the benzophenone crosslinker and expands our toolbox of chemical probes for the mapping of PPIs in their native cellular environment.

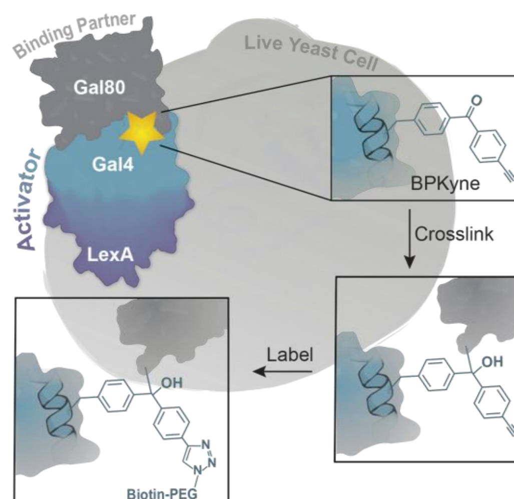

Figure 1. Experimental scheme of BPKyne crosslinking and bioconjugation using the copper-catalyzed alkyne-azide Huisgen cycloaddition (CuAAC).

Transient and moderate affinity protein-protein interactions (PPIs) play crucial roles across cellular processes, but are often intractable to characterize in their native environments.[1] In the case of transcriptional initiation, for example, the complexes formed between transcriptional activators and coactivators are instrumental in the proper assembly of the transcriptional machinery, yet the necessarily transient interactions have frustrated efforts to identify specific protein pairings.[2] A breakthrough was realized with genetic code expansion via amber nonsense suppression, enabling the site specific incorporation of photo-activatable amino acids into protein partners in living cells for covalent capture experiments.[3] Over the last several years we have demonstrated that the photo-activatable unnatural amino acid (UAA) *p*-benzoyl-L-phenylalanine (pBpa) effectively captures even the modest affinity interactions between DNA-bound transcriptional activators and their binding partners *in vivo*, allowing the creation of a detailed map of several key PPIs that define transcriptional activation.[4] Nonetheless, these experiments are technically challenging and require several steps of isolation and purification post-crosslinking. This is typically accomplished through immunoprecipitation and/or affinity purification of the protein of interest or its binding partners and can vary significantly in efficiency, especially for low abundance proteins. Further, immunoprecipitation requires effective antibodies and/or genetic incorporation of epitope tags into the protein of interest.[5] Therefore, there is a real need for additional methods for isolation of *in vivo* crosslinked proteins.

To address this, we hypothesized that a bifunctional UAA containing both a photo-activatable group and a moiety that would enable post-crosslinking derivatization would facilitate detection, isolation, and/or identification of PPIs in the context of the native cellular environment. The bifunctional pBpa derivative 4-ethynyl-*p*-benzoyl-L-phenylalanine (BPKyne) contains an alkynyl moiety that can be functionalized post-crosslinking using copper-mediated azide-alkyne Huisgen cycloaddition (CuAAC) (Figure 1).[6] Originally reported for use in synthetic peptides and *in vitro* crosslinking, we sought to incorporate it into proteins *in vivo* using nonsense suppression, and functionalization of the alkyne handle post-crosslinking would enable isolation and visualization of crosslinked products.[7] Here we describe the first reported incorporation of BPKyne in live cells using the prototypical yeast Gal4-Gal80 transcriptional complex.[4a, 8] We further demonstrate that alkyne functionalization enables isolation and purification of *in vivo* crosslinked products.

At the outset a need for a more generally accessible synthetic route to BPKyne was noted (Scheme 1). Commercially available 4-iodo-L-phenylalanine (**1**) was first protected as the Boc methyl ester **2**. To assemble the benzophenone core of **3**, we utilized an air-tolerant carbonylative Suzuki-Miyaura coupling in which carbon monoxide is generate *in situ* from the palladium-catalyzed decomposition of 9-methylfluorene-9-carbonyl chloride (COgen).[9] This route avoids the use of pyrophoric reagents such as *tert*-butyl lithium that were required for assembling the benzophenone moiety in the previous synthesis.[7] Next, the

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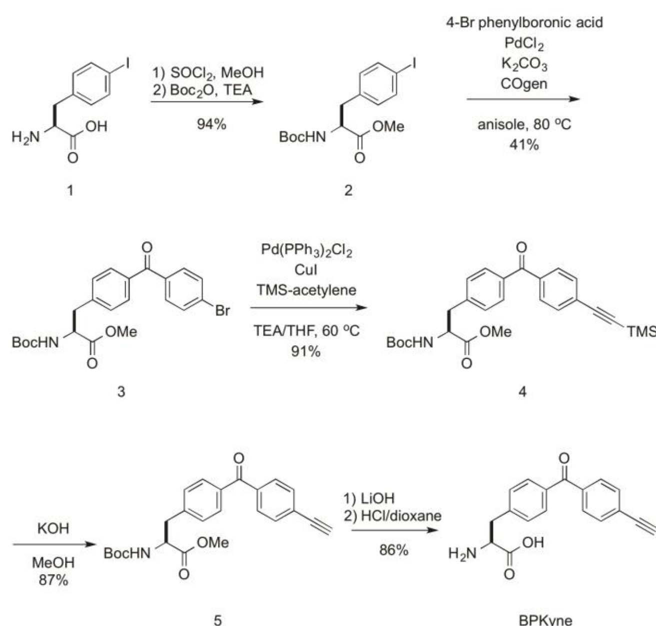
alkyne moiety was installed via a Sonagashira coupling to give the TMS protected alkynyl-benzophenone **4**. Removal of the TMS protecting group under basic conditions generated the alkynyl-benzophenone **5**. Finally, hydrolysis of the methyl ester protecting group followed by Boc deprotection yielded BPKyne.



Scheme 1: BPKyne synthetic route

Previously, our lab has used the well-established *Escherichia coli* tyrosyl tRNA/synthetase pair ($tRNA^{Tyr}_{CUA}$-TyrRS) to incorporate pBpa into proteins in their native cellular environment using nonsense suppression.[4] It has recently been shown that the bioorthogonal tRNA synthetases can incorporate analogs of the cognate UAA without further alterations in some cases.[3c, 10] An examination of the crystal structure of the *E. coli* tyrosyl tRNA synthetase suggested that due to the small van der Waals radius of the alkynyl moiety, BPKyne should fit in the active site and be incorporated without any modifications to the synthetase.[3c] To test this, we compared the expression levels of the chimeric transcriptional activator LexA+Gal4 when pBpa or BPKyne was incorporated at position 849 within the Gal4 transcriptional activation domain (TAD) (Figure 2A). Using identical conditions, the expression level of BPKyne incorporated LexA+Gal4 was found to be within 10% of that observed with pBpa, illustrating the substrate flexibility of the tRNA/synthetase pair to pBpa analogs (Figure 2B). Additionally, incorporation of BPKyne does not alter LexA+Gal4-mediated transcriptional activation (See Supporting Information S1).

Next, we evaluated the effect of the alkynyl moiety on the photochemical reactivity of the benzophenone through *in vivo* photo-crosslinking experiments using the LexA+Gal4 transcriptional activator. Live yeast expressing LexA+Gal4 with either pBpa or BPKyne incorporated at position 849 were irradiated at 365 nm to capture Gal4's endogenous binding partners. Upon lysis and Western blot analysis probing for the Flag-tagged LexA+Gal4 activator, several crosslinked products were captured by both photo-crosslinkers. Both molecules captured a distinct product around 80 kDa, consistent with the Gal4-Gal80 complex (Figure 2C).[4a] To confirm this, a 6x-Myc-tagged Gal80 construct was transformed into live yeast with the UAA-incorporated LexA+Gal4 fusion protein. After irradiation, lysis, and Western blot analysis probing for the Myc-tagged Gal80 protein, both pBpa and BPKyne captured a Gal4-Gal80 crosslinked product (Figure 2D). When comparing the amount of crosslinked Gal4-Gal80, BPKyne's crosslinking efficiency is approximately two-thirds that of the parent molecule, at least in this context. A small decrease was expected due to the stereoelectronic influence of the triple bond [11] (Figure 2C and D).

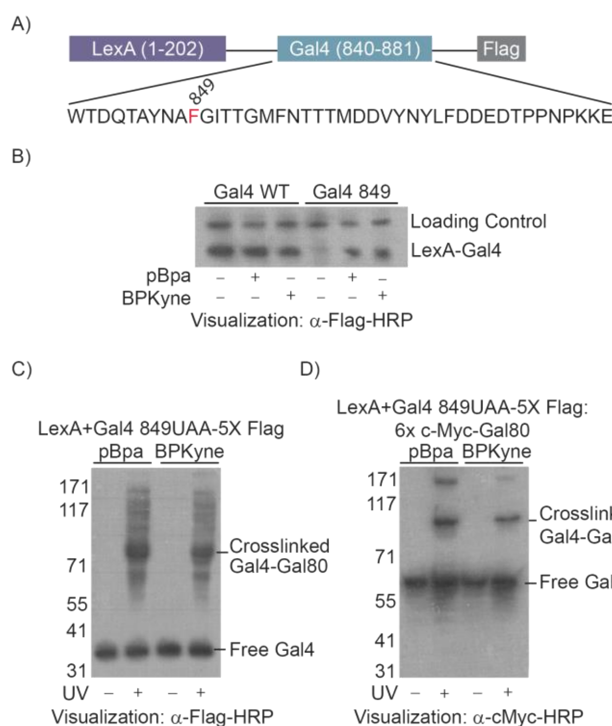


Figure 2. Analysis of BPKyne incorporation and crosslinking in the LexA+Gal4 849TAG-Flag protein. A) Plasmid encoding the LexA DNA-binding domain (DBD) fused to the Gal4 transcriptional activation domain (TAD) and a Flag tag were constructed. Position 849 (in red) was mutated to the amber stop codon for pBpa and BPKyne incorporation. B) BPKyne incorporation was compared to pBpa in the 849 position of Gal4 using the *E. coli* tyrosyl tRNA/tRNA synthetase pair ($tRNA^{Tyr}_{CUA}$-TyrRS) in the presence or absence of 1mM pBpa or BPKyne. Expression levels of LexA+Gal4 849UAA mutants relative to LexA+Gal4 WT were quantified using ImageJ.[12] C) BPKyne functions in covalent chemical capture. BPKyne captured several of Gal4's endogenous protein partners including, Gal80 (crosslinked band around 80 kDa). D) BPKyne captures the Myc-Gal80 interaction with Gal4 to confirm the Gal80 crosslinked band at 80 kDa. The band at around 90 kDa corresponding to the crosslinked LexA-Gal4 and c-Myc Gal80 is only observed after UV irradiation. Crosslinking yield of LexA+Gal4 849BPKyne-Gal80 relative to LexA+Gal4 F849pBpa-Gal80 was quantified using ImageJ.

Once incorporation and crosslinking were confirmed, the bioconjugation capability of BPKyne was characterized post-crosslinking and was compared to traditional immunological methods for the isolation of crosslinked products.[4a] Towards this end, the UAA was incorporated into a Gal80 construct with position 245 mutated to the amber stop codon. This site is located at the outer edge of the Gal4 binding interface, and therefore when this construct is irradiated only the Gal80-Gal4 complex should be captured, allowing for a single interaction to be visualized. To demonstrate that the bioorthogonal alkyne

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handle of BPKyne could be functionalized post-crosslinking, live yeast expressing Flag-tagged LexA+Gal4 and Myc-tagged Gal80 with pBpa or BPKyne incorporated at position 245 were grown under glucose conditions and irradiated to capture the Gal80-Gal4 binding event. After lysis, biotin-PEG$_3$-azide was conjugated to the BPKyne incorporated Gal80 species via a Huisgen cycloaddition in whole cell lysate using copper (II) sulphate, THPTA, and sodium ascorbate at 37°C (Figure 3A).[6] After 2 hours, the biotin-conjugated proteins were isolated using neutravadin magnetic beads and analyzed by Western blot probing for the Myc-tagged Gal80 species (Figure 3B). With this strategy the Lexa+Gal4-Gal80 complex is only observed for BPKyne incorporated proteins that have been irradiated and functionalized with the biotin-azide probe demonstrating the ability of the bioorthogonal alkyne handle to be specifically labeled. As a comparison, traditional immunological techniques were used to isolate the pBpa-containing Gal80-Gal4 crosslinked complex and visualized by Western blot. Importantly, when visualized by Western blot using the Myc-HRP antibody, less background is seen when BPKyne containing samples isolated through CuAAC and neutravadin pull-down are compared to pBpa containing proteins immunoprecipitated with Myc, which results in nonspecific isolation of all protein containing an endogenous Myc epitope (Figure 3B and S2). These experiments illustrate the advantages of the bifunctional BPKyne molecule, which captures specific PPIs upon irradiation and allows for isolation of these interactions from their cellular environment post-functionalization.

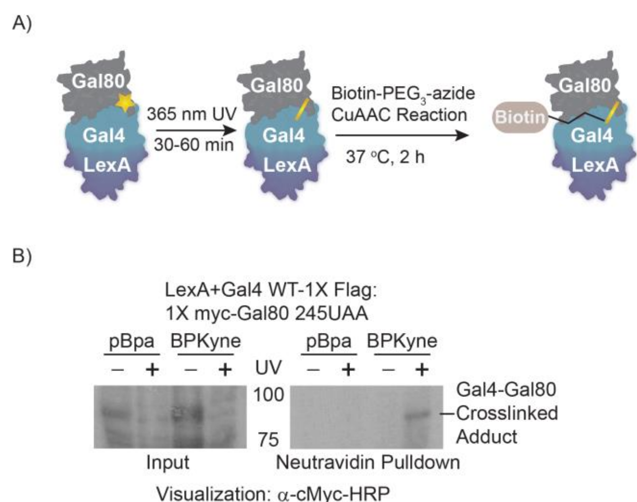

Figure 3. Analysis of BPKyne bioconjugation using Huisgen copper-catalyzed alkyne-azide cycloaddition (CuAAC). A) Experimental workflow of isolation of Gal80 245BPKyne-Gal4 crosslinked products from yeast cells. B) Biotinylation of Gal4-Gal80 crosslinked product through CuAAC cycloaddition. The BPKyne incorporated Gal80-Gal4 crosslinked product was isolated from solution using CuAAC and neutravadin magnetic beads and analyzed by Western blot (α-Myc). The Gal4-Gal80 crosslinked product is only isolated in the presence of BPKyne and UV when conjugated to the biotin probe. [See Supplemental Figure S4 for expression of Myc-Gal80 245UAA]

Here we have demonstrated the first incorporation of the bifunctional UAA, BPKyne, into live yeast cells using the *E. coli* tyrosyl tRNA/synthetase system and have illustrated the utility of BPKyne for the isolation of crosslinked products from their native environment. Utilizing the Gal4 and Gal80 yeast proteins we have shown that BPKyne is incorporated with similar expression yields compared to pBpa without requiring further mutagenesis. Along with the similar crosslinking yield compared to pBpa, we have illustrated that BPKyne incorporated proteins can be isolated from whole cell lysate after functionalization with a biotinylated azide probe. While we used Western blotting for visualization in this proof of principle study, mass spectrometry could also be used for characterization of isolated crosslinked adducts. This strategy also enables the capture and isolation of PPIs for which antibodies are not efficient or available or when genetically encoded epitope tags, such as Myc or flag, cannot be appended without impairment of protein structure or function. The bioorthogonal alkyne handle enables the direct labeling of crosslinked PPIs of interest, which will be particularly advantageous in the discovery of novel PPIs.

Experimental Section

For full experimental details see Supporting Information.

Acknowledgements

We are grateful to the NSF CHE 1412759 for support of this work. We thank Professor B. Martin and Dr. C. Custer for insightful discussion and Dr. A. Dugan for construction of the LexA+Gal4 WT-5X Flag LexA+Gal4 849TAG-5X Flag expression plasmids.

Keywords: unnatural amino acids • photo-crosslinking • protein-protein interactions • bioorthogonal labelling • Click chemistry

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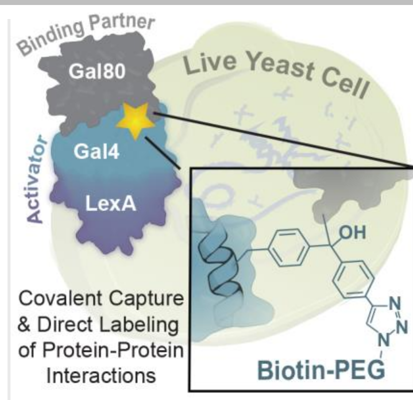
Using the bifunctional unnatural amino acid, BPKyne, we have developed a strategy to capture and directly label transient protein-protein interactions (PPIs) in their native environment. Click chemical-functionalization post-crosslinking with a biotin-azide probe enabled the isolation of transcriptional protein complexes from yeast cells. This amino acid will expand the toolbox for the discovery of novel PPIs in live cells.



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