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Very Important Paper

A Bifunctional Amino Acid Enables Both Covalent Chemical Capture and Isolation of in Vivo Protein–Protein Interactions

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In vivo covalent chemical capture by using photoactivatable 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 photoactivatable UAA pBpa and effectively captures the Gal4–Gal80 transcriptional complex. Post-crosslinking, the Gal4–Gal80 adduct was directly labeled by treatment of the alkyne handle with a biotin-azide probe; this 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 mapping PPIs in their native cellular environment.

Transient and moderate-affinity protein–protein interactions (PPIs) play crucial roles across cellular processes, but are often difficult 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 through amber nonsense suppression; this enabled the site-specific incorporation of photoactivatable amino acids into protein partners in living cells for covalent capture experiments.[3] Over the last several years, we have demonstrated that the photoactivatable unnatural amino acid (UAA) *p*-benzoyl-L-phenylalanine (pBpa) effectively captures even modest-affinity interactions between DNA-bound transcriptional activators and their binding partners in vivo, thus 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 proteins that are low in abundance. Furthermore, 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 the isolation of in vivo crosslinked proteins.

We hypothesized that a bifunctional UAA containing both a photoactivatable group and a moiety that would enable post-crosslinking derivatization would facilitate the 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 by using copper-mediated azide–alkyne Huisgen cycloaddition (CuAAC; Figure 1).[6] Although BPKyne was originally reported for use in synthetic peptides and in vitro crosslinking, we sought to incorporate it into proteins in vivo by using nonsense suppression; functionalization of the alkyne handle post-crosslinking would enable the isolation and visualization of crosslinked products.[7] Here we describe the first reported in-

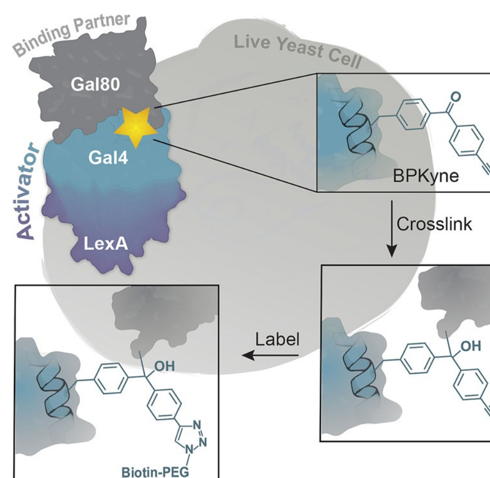

Figure 1. Experimental scheme of BPKyne crosslinking and bioconjugation by CuAAC.

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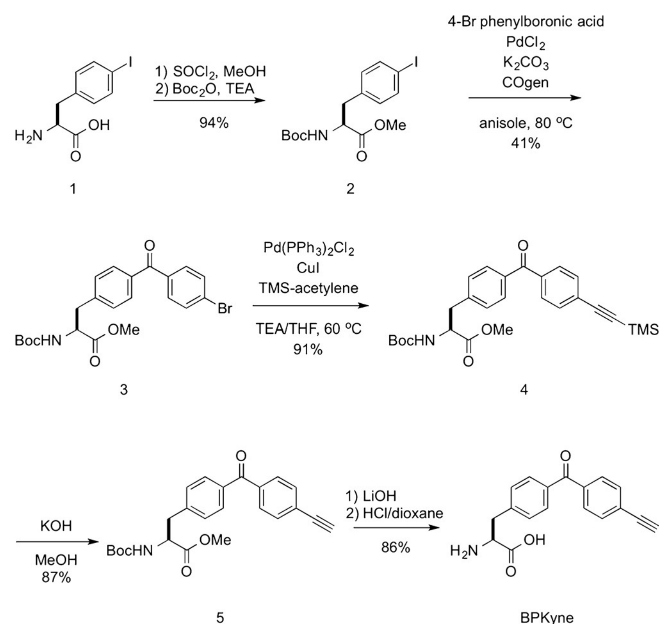
corporation of BPKyne in live cells by using the prototypical yeast Gal4–Gal80 transcriptional complex.[4a,8] We further demonstrate that alkyne functionalization enables isolation and purification of in vivo crosslinked products.

At the outset, a need for a more generally accessible synthetic route to BPKyne was noted. Commercially available 4-iodo-L-phenylalanine (**1**) was first protected as the Boc methyl ester **2** (Scheme 1). 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, which were required for assembling the benzophenone moiety in the previous synthesis.[7] Next, the alkyne moiety was installed by Sonogashira coupling to give the TMS-protected alkynylbenzophenone **4**. Removal of the TMS protecting group under basic conditions generated alkynylbenzophenone **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 by using nonsense suppression.[4] It has been shown that the bio-orthogonal tRNA synthetases can incorporate analogues 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 the need for 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). Under identical conditions, the expression level of LexA+Gal4 when BPKyne was incorporated was found to be within 10% of that observed with pBpa, thus illustrating the substrate flexibility of the tRNA/synthetase pair to pBpa analogues (Figure 2B). Additionally, incorporation of BPKyne does not alter LexA+Gal4-mediated transcriptional activation (see the 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 with the LexA+Gal4 transcriptional activator. Live yeast expressing LexA+Gal4 with either pBpa or BPKyne incorporated at position 849 was 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

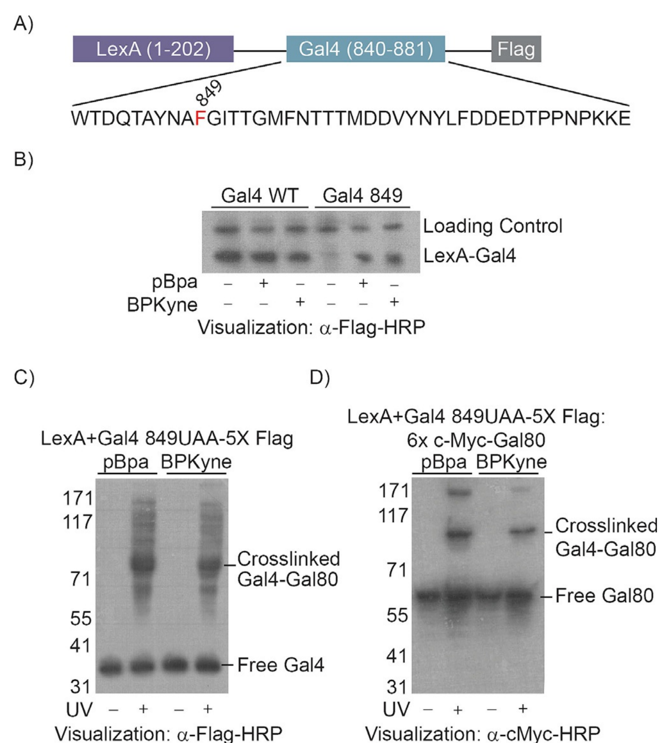


Figure 2. Analysis of BPKyne incorporation and crosslinking in the LexA+Gal4 849TAG-Flag protein. A) A Plasmid encoding the LexA DNA-binding domain (DBD) fused to the Gal4 transcriptional activation domain (TAD) and a Flag tag was constructed. Position 849 (in red) was mutated to the amber stop codon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

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distinct product of approximately 80 kDa, consistent with the Gal4–Gal80 complex (Figure 2C).[4a] To confirm this, a Myc_6-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).

Once incorporation and crosslinking were confirmed, the bioconjugation capability of BPKyne was characterized post-crosslinking and was compared to that of traditional immunological methods for the isolation of crosslinked products.[4a] 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, therefore when this construct is irradiated only the Gal80–Gal4 complex should be captured; this allows a single interaction to be visualized. To demonstrate that the bioorthogonal alkyne 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 was 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 through a Huisgen cycloaddition in whole-cell lysate by using copper(II) sulfate, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), and sodium ascorbate at 37 °C (Figure 3A).[6] After 2 hours, the biotin-conjugated proteins were isolated on neutravidin 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, thus 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, which was visualized by western blot. Importantly, when visualized by western blot with the Myc-HRP antibody, less background is seen when BPKyne-containing samples isolated through CuAAC and neutravidin pull-down are compared to pBpa-containing proteins immunoprecipitated with Myc; this results in nonspecific isolation of all protein containing an endogenous Myc epitope (Figures 3B and S2). These experiments illustrate the advantages of the bifunctional BPKyne molecule, which captures specific PPIs upon irradiation and allows them to be isolated from their cellular environment post-functionalization.

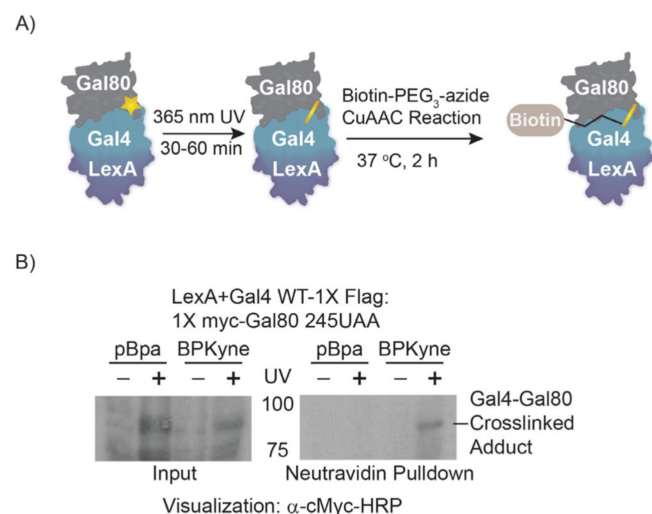

Figure 3. Analysis of BPKyne bioconjugation by CuAAC. A) Experimental workflow for isolation of Gal80 245BPKyne–Gal4 crosslinked products from yeast cells. B) Biotinylation of Gal4–Gal80 crosslinked product through CuAAC cycloaddition. The BPKyne-incorporated Gal80–Gal4 crosslinked product was isolated from solution by using CuAAC and neutravidin 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 Figure S4 for expression of Myc-Gal80 245UAA.)

Here we have demonstrated the first incorporation of the bifunctional UAA BPKyne into live yeast cells by 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 to pBpa without requiring further mutagenesis. Along with the similar crosslinking yield compared to pBpa, we have illustrated that BPKyne-containing proteins can be isolated from whole-cell lysate after functionalization with a biotinylated azide probe. Although we used western blotting for visualization in this proof-of-principle study, mass spectrometry could also be used to characterize 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 impairing 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.

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Keywords: bioorthogonal labeling · click chemistry · photocrosslinking · protein–protein interactions · unnatural amino acids

[1] a) A. L. Hopkins, C. R. Groom, *Nat. Rev. Drug Discovery* **2002**, *1*, 727–730; b) S. Surade, T. L. Blundell, *Chem. Biol.* **2012**, *19*, 42–50.
[2] a) A. D. Thompson, A. Dugan, J. E. Gestwicki, A. K. Mapp, *ACS Chem. Biol.* **2012**, *7*, 1311–1320; b) A. K. Mapp, A. Z. Ansari, *ACS Chem. Biol.*

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2007, *2*, 62–75; c) J.-F. Rual, K. Venkatesan, T. Hao, T. Hirozane-Kishikawa, A. Dricot, N. Li, G. F. Berriz, F. D. Gibbons, M. Dreze, N. Ayivi-Guedehoussou, et al., *Nature* **2005**, *437*, 1173–1178; d) J. R. Perkins, I. Diboun, B. H. Dessailly, J. G. Lees, C. Orengo, *Structure* **2010**, *18*, 1233–1243; e) T. Berggård, S. Linse, P. James, *Proteomics* **2007**, *7*, 2833–2842.

[3] a) J. W. Chin, P. G. Schultz, *ChemBioChem* **2002**, *3*, 1135–1137; b) J. W. Chin, T. A. Cropp, J. C. Anderson, M. Mukherji, Z. Zhang, P. G. Schultz, *Science* **2003**, *301*, 964–967; c) W. Liu, L. Alfonta, A. V. Mack, P. G. Schultz, *Angew. Chem. Int. Ed.* **2007**, *46*, 6073–6075; *Angew. Chem.* **2007**, *119*, 6185–6187; d) Q. Wang, L. Wang, *J. Am. Chem. Soc.* **2008**, *130*, 6066–6067; e) T. C. Lee, M. Kang, C. H. Kim, P. G. Schultz, E. Chapman, A. A. Deniz, *ChemBioChem* **2016**, *17*, 981–984; f) A. Yamaguchi, T. Matsuda, K. Ohtake, T. Yanagisawa, S. Yokoyama, Y. Fujiwara, T. Watanabe, T. Hohsaka, K. Sakamoto, *Bioconjugate Chem.* **2016**, *27*, 198–206; g) N. Hino, Y. Okazaki, T. Kobayashi, A. Hayashi, K. Sakamoto, S. Yokoyama, *Nat. Methods* **2005**, *2*, 201–206.

[4] a) C. Y. Majmudar, L. W. Lee, J. K. Lancia, A. Nwokoye, Q. Wang, A. M. Wangs, L. Wang, A. K. Mapp, *J. Am. Chem. Soc.* **2009**, *131*, 14240–14242; b) M. Krishnamurthy, A. Dugan, A. Nwokoye, Y.-H. Fung, J. K. Lancia, C. Y. Majmudar, A. K. Mapp, *ACS Chem. Biol.* **2011**, *6*, 1321–1326; c) A. Dugan, R. Pricer, M. Katz, A. K. Mapp, *Protein Sci.* **2016**, *25*, 1371–1377.

[5] C. E. Fritze, T. R. Anderson in *Methods in Enzymology, Vol. 327: Applications of Chimeric Genes and Hybrid Proteins–Part B: Cell Biology and Physiology* (Eds.: J. Thorner, S. D. Emr, J. N. Abelson), Academic Press, San Diego, **2000**, pp. 3–16.

[6] S. I. Presolski, V. P. Hong, M. G. Finn in *Current Protocols in Chemical Biology*, Wiley, Hoboken, **2009**.

[7] Y. Chen, Y. Wu, P. Henklein, X. Li, K. P. Hofmann, K. Nakanishi, O. P. Ernst, *Chem. Eur. J.* **2010**, *16*, 7389–7394.

[8] a) J. B. Thoden, L. A. Ryan, R. J. Reece, H. M. Holden, *J. Biol. Chem.* **2008**, *283*, 30266–30272; b) A. Z. Ansari, R. J. Reece, M. Ptashne, *Proc. Natl. Acad. Sci. USa* **1998**, *95*, 13543–13548.

[9] A. Ahlburg, A. T. Lindhardt, R. H. Taaning, A. E. Modvig, T. Skrydstrup, *J. Org. Chem.* **2013**, *78*, 10310–10318.

[10] a) A. L. Stokes, S. J. Miyake-Stoner, J. C. Peeler, D. P. Nguyen, R. P. Hammer, R. A. Mehl, *Mol. BioSyst.* **2009**, *5*, 1032; b) Y.-S. Wang, X. Fang, A. L. Wallace, B. Wu, W. R. Liu, *J. Am. Chem. Soc.* **2012**, *134*, 2950–2953; c) D. D. Young, S. Jockush, N. J. Turro, P. G. Schultz, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 7502–7504.

[11] G. Dorman, G. D. Prestwich, *Biochemistry* **1994**, *33*, 5661–5673.

[12] J. K. Lancia, A. Nwokoye, A. Dugan, C. Joiner, R. Pricer, A. K. Mapp, *Biopolymers* **2014**, *101*, 391–397.

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