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Communication

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Two-Step Protein Labeling Utilizing Lipoic Acid Ligase and Sonogashira Cross-Coupling

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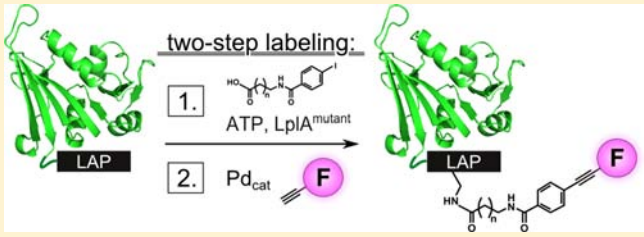
Supporting Information

ABSTRACT: Labeling proteins in their natural settings with fluorescent proteins or protein tags often leads to problems. Despite the high specificity, these methods influence the natural functions due to the rather large size of the proteins used. Here we present a two-step labeling procedure for the attachment of various fluorescent probes to a small peptide sequence (13 amino acids) using enzyme-mediated peptide labeling in combination with palladium-catalyzed Sonogashira cross-coupling. We identified *p*-iodophenyl derivatives from a small library that can be covalently attached to a lysine residue within a specific 13-amino-acid peptide sequence by *Escherichia coli* lipoic acid ligase A (LplA). The derivatization with *p*-iodophenyl subsequently served as a reactive handle for bioorthogonal transition metal-catalyzed Sonogashira cross-coupling with alkyne-functionalized fluorophores on both the peptide as well as on the protein level. Our two-step labeling strategy combines high selectivity of enzyme-mediated labeling with the chemoselectivity of palladium-catalyzed Sonogashira cross-coupling.



INTRODUCTION

Cellular processes are based on the complex interplay of biopolymers such as proteins, nucleic acids, polysaccharides, and small molecule metabolites. To unravel the role and function of individual biomolecules within their cellular context, it is important to complete our understanding in cell and organismal biology. Most promising investigations of vital cellular processes are performed in the context of living cells or even whole organisms due to the complexity of interactions within sophisticated networks of biomolecules, ions, and metabolites. However, the obvious challenge is the selective introduction of reporter probes to elucidate the role and function of a specific biomolecule within its native environment. Numerous different methods have been reported to endow biomolecules with reporter tags, facilitating the investigation within their natural complex cellular environment.[1−5] Over the past decade, highly selective chemical reactions, nonperturbing to the natural environment, have become powerful tools for the selective, covalent attachment of probes to specific biomolecules. In recent studies, it has been shown that the palladium catalyzed cross-coupling reactions like the Suzuki-Miyaura,[6−12] Heck,[13,14] and Sonogashira coupling[15−22] also add to the toolbox of bioorthogonal chemistry.[23−25] Due to the overall mild reaction conditions and the tolerance over other functional groups, the cross-coupling reactions find widespread application in organic chemistry synthesis, first and foremost in carbon−carbon bond formation.[26] Furthermore, cross-coupling chemistry has extensively been investigated under aqueous conditions for its possible application in green chemistry.[27−29] The functional groups necessary for most cross-coupling reactions can rarely be found in nature. Therefore, the mild reaction conditions and the chemical versatility make palladium-catalyzed cross-coupling particularly attractive for the selective labeling of biomolecules.

However, the remaining challenge for applications of cross-coupling chemistry for biomolecule labeling in complex biological settings is the selective incorporation of addressable functional groups into the biomolecule of interest. One current strategy presents the introduction of unnatural functional groups into proteins by incorporation of unnatural amino acids (UAA).[30,31] Li et al. recently demonstrated the incorporation of alkyne handles into a virulence protein in *Shigella* using a pyrrolysine-based system.[18] The alkyne was subsequently labeled with a fluorophore, applying palladium-mediated Sonogashira cross-coupling within live bacteria. However, despite their excellent selectivity, methods which make use of UAA for live cell labeling face some difficulties. Although well-established for use in bacteria and yeast, the incorporation of UAA into proteins in mammalian cells is still considerably more challenging.[32−34]

Here we present a general strategy for the selective incorporation of cross-coupling reactive functional handles into proteins based on enzyme-mediated peptide labeling utilizing the lipoic acid ligase (LplA) from *Escherichia coli* (*E. coli*).[35] LplA has previously been reported to exhibit a certain

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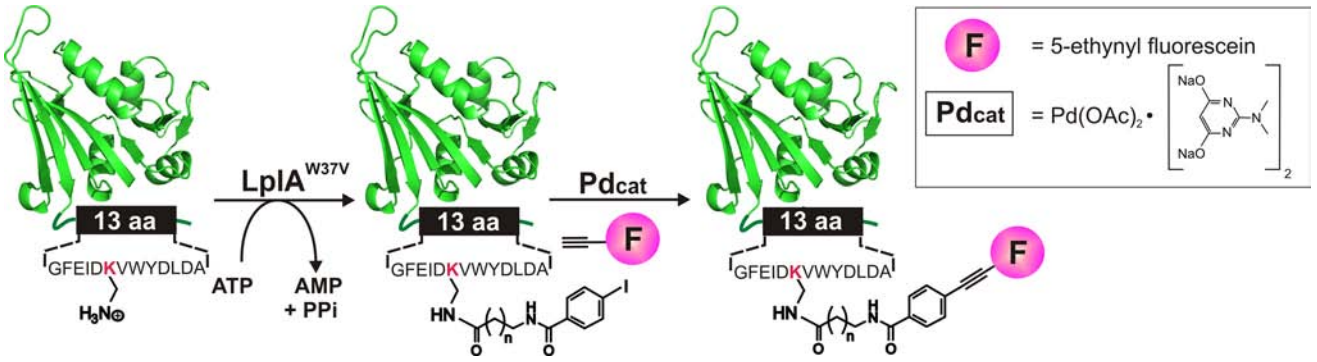
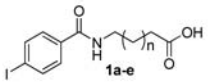


Figure 1. Two-step labeling procedure of the model protein *E. coli* eDHFR, designed with an N-terminal 13 amino acid LAP sequence. In a first step, the *p*-iodophenyl carboxylic acid is coupled to the amino group of the lysine side chain by LplA^{W37V} as a chemical handle which is afterward specifically labeled with an ethynyle-modified fluorescent dye by palladium catalyzed Sonogashira cross-coupling.

Table 1. Screening of LplA Mutants for Their Tolerance of *p*-Iodophenyl Derivatives 1a−e as Substrate for Ligation to the 13 aa LAP Peptide

Substrate[a]	substrate	LplA^{wt}	LplA^{W37V}	LplA$^{W37V, E20A}$	LplA$^{W37V, E20L}$	LplA^{H79A}
	n = 1, 1a	< 1	9	5	1	< 1
	n = 2, 1b	7	100	21	12	1
	n = 3, 1c	< 1	100	13	3	< 1
	n = 4, 1d	< 1	3	1	< 1	< 1
	n = 5, 1e	< 1	3	< 1	< 1	< 1

[a]The turnover is given in %, calculated by the integrated ratio of LAP-**1b** and the sum of LAP-**1b** and LAP. All measurements were done in triplicate. Minor changes of the absorption due to product formation were neglected.

substrate promiscuity accepting other carboxylic acid derivatives besides the natural lipoic acid substrate.[36] One example is the redirection of the LplA to selectively attach alkyl azides to an engineered 13-amino-acid lipoic acid acceptor peptide sequence (LAP).[37,38] The azide functional group was subsequently derivatized with fluorescent probes by CuAAC or SPAAC. Overall, the labeling of proteins for both intracellular[39−42] and extracellular[37,40,41,43] labeling using CuAAC,[44,45] SPAAC,[46] hydrazone formation,[47] and inverse electron demand [4 + 2]-cyclo-addition[42] was demonstrated. The major advantage of LplA-mediated peptide labeling over the established labeling strategies[48] like SNAP/CLIP tag,[49,50] HaloTag,[51] and TMP-tag[52−57] is the small size of the LAP tag, which is minimally disruptive and therefore does not interfere with the protein function. We sought to use versatile LplA-mediated labeling to introduce *p*-iodophenyl derivatives as functional handles for subsequent palladium mediated Sonogashira cross-coupling. Here we demonstrate such a two-step protein labeling strategy for the labeling of peptides as well as proteins (Figure 1).

Based on the previously reported substrate selectivity of LplA, we created four LplA mutants which we screened with a set of five different *p*-iodophenyl derivatives in a peptide based in vitro assay. We identified the LplA mutant-*p*-iodophenyl substrate pair that is best suited for the first step of the labeling procedure. For enzymatic ligation reactions in the first step, 2-fold excess of the *p*-iodophenyl substrate was found to be just sufficient for quantitative product formation and minimal background levels of nonreacted species. We then demonstrated the efficient labeling of the 13 aa LAP with an alkyne-functionalized fluorophore using Sonogashira cross-coupling in a second step. As a catalyst, we chose a robust aminopyridine−palladium(II) complex, which has previously been reported for copper-free Sonogashira cross-coupling under aqueous conditions.[19] Finally, we demonstrated the in vitro labeling of a LAP tagged model protein, namely, *E. coli* dihydrofolate reductase using the two-step labeling procedure.

RESULTS AND DISCUSSION

Synthesis of *p*-Iodophenyl Carboxylic Acid Derivatives. We synthesized five different *p*-iodophenyl carboxylic acid derivatives (**1a−e**) varying in the length of the aliphatic linker of the *n*-amino carboxylic acid attached to *p*-iodobenzoic acid via an amide bond. Our choice of linker lengths between 3 and 7 carbon atoms was initially based on the natural substrate for LplA, namely, lipoic acid, but particularly on the previously reported substrate analogues *n*-azide carboxylic acids,[37] *trans*-cyclooctenes,[42] and coumarin derivatives[39] that have been shown to be accepted as substrates by the lipoic acid ligase or corresponding mutants thereof.[39,42] The *p*-iodophenyl carboxylic acid derivatives were synthesized from *p*-iodophenyl benzoic acid which was converted to the activated NHS-ester (**3**) and then coupled to the respective *n*-amino carboxylic acids varying in linker length to yield the compounds **1a−e** (see Supporting Information for structural details).

Engineering an Aryl-Iodo Ligase. To specifically label peptides or proteins with various fluorophores, we applied a two-step labeling procedure; utilizing *E. coli* lipoic acid ligase (LplA) for the specific introduction of *p*-iodophenyl carboxylic acid derivatives in a first step, the incorporated handles allowing bioorthogonal Sonogashira cross-coupling with alkyne-labeled fluorophores in a second step. We prepared four different LplA-constructs with mutations in their active sites to increase the volume of the binding pocket for the artificial *p*-iodophenyl carboxylic acid substrates. In previous work, W37 had been described as an important gatekeeper,[39−41,43] which, when

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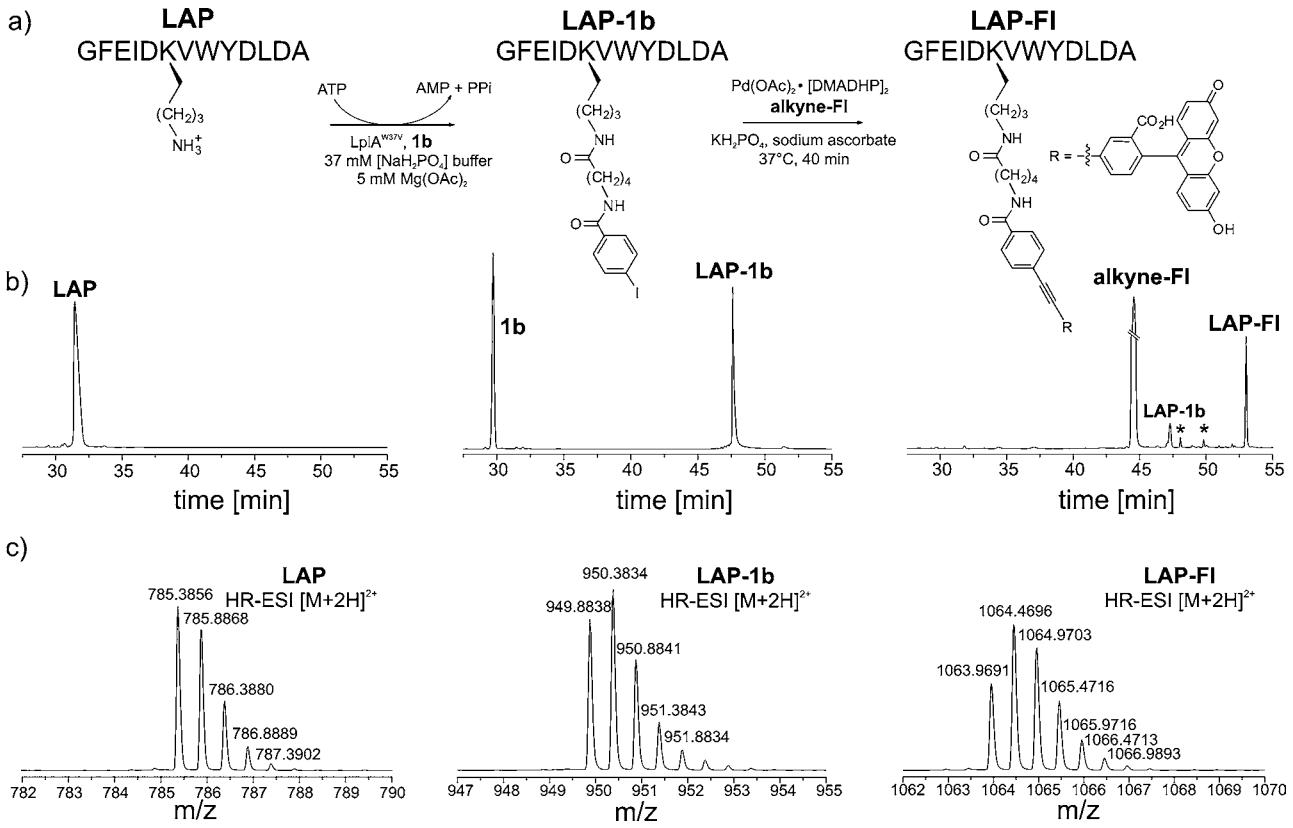

Figure 2. (A) Two-step labeling procedure of LAP. LplA^{W37V}-mediated coupling of LAP to *p*-iodophenyl compound **1b** was followed by palladium catalyzed Sonogashira cross-coupling reaction of the coupled product LAP-**1b** with alkyne-Fl in KH_2PO_4-buffered aqueous settings to obtain LAP-Fl. (B) Two-step labeling process was followed by HPLC (detection at λ = 280 nm). LAP was monitored before and after enzymatic conversion in the presence of the *p*-iodophenyl compound **1b** to give the coupled product LAP-**1b** (middle). The conversion of the latter with alkyne-Fl in KH_2PO_4-buffered aqueous settings to the final ligation product LAP-Fl is shown in the right chromatogram. The asterisks highlight the signals from the palladium ligand. (C) Monitoring of the two-step coupling reaction by LC-MS analysis, identifying LAP (left), LAP-**1b** (middle), and the final coupling product LAP-Fl (right).

mutated to a smaller amino acid, led to acceptance of coumarins[39] and *trans*-cyclooctenes as substrates.[42] From saturation mutagenesis studies, the LplA^{W37V} mutant was identified as ideally suitable for the ligation of aryl azides to the acceptor peptide. Notably, the aryl azide substrate applied in their work is structurally very similar to the *p*-iodophenyl derivatives used in the present study. Therefore, we prepared the W37V and H79A mutants of LplA by side-directed mutagenesis. Taking into consideration the relatively large van der Waals radius (2.2 Å[58,59]) of the iodo-substituted in *p*-position of the aryl, we also prepared the double mutants LplA$^{W37V/E20A}$ and LplA$^{W37V/E20L}$.

Screening of LplA Mutants for Ligation of *p*-Iodophenyl Carboxylic Acid Derivatives to LAP. First, we aimed to identify the optimal LplA mutant-*p*-iodo pair for the efficient ligation of *p*-iodophenyl carboxylic acid to the 13-amino-acid peptide LAP. For this, we examined the ligation efficiency of the *p*-iodophenyl carboxylic acids **1a**−**e** to the acceptor peptide LAP with each of the four LplA ligase mutants as well as the wild type LplA ligase. Reaction conditions were chosen to be compatible with physiological conditions at 37 °C. The enzymatic reaction was terminated by the addition of EDTA after 45 min and the ligation product formation was monitored by HPLC (Table 1 and Supporting Information Figure S3). For the wild type enzyme, we did not observe significant product formation for any of the tested compounds **1a**−**e**. Generally, significant turnover was solely observed for the compounds **1b** and **1c**. For both of these substrates we observed full turnover with the LplA^{W37V} mutant, yielding quantitative product formation. This supports the previously reported observation from other groups regarding the importance of tryptophan 37 as gatekeeper within the LplA binding site.[39−41,43] Notably, the introduction of additional mutations (W37V/E20A or W37V/E20L) decreased ligation rates under the applied assay conditions. The mutant H79A did not show noteworthy product formation for any of the substrates **1a**−**e**. From our experiments, we conclude that the LplA^{W37V} mutant is suitable to accommodate the size and shape of the *p*-iodophenyl substrates **1b** and **1c**. We selected the LplA^{W37V}-mediated ligation reaction with substrate **1b** due to the better solubility over that of **1c** in water and applied this setup for all subsequent experiments with 2-fold excess of **1b** over LAP, which was found to be sufficient for quantitative product formation (see Supporting Information Figure S6).

Palladium-Catalyzed Sonogashira Cross-Coupling of the *p*-Iodophenyl Compound with 5-Ethynylefluorescein (Alkyne-Fl) in Aqueous Buffer. Next, we identified optimal conditions for the second modification step of the *p*-iodophenyl handle using palladium-catalyzed Sonogashira cross-coupling. We set up a model reaction to determine the suitable conditions for this individual coupling step to transfer them to the general two-step labeling procedure on LAP. For this, we tested the coupling of *p*-iodophenyl compound **1b** to 5-ethynylefluorescein (alkyne-Fl) using palladium-mediated

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Sonogashira cross-coupling. Reaction conditions were adapted from previous reports[19] in KH_2PO_4-buffer with sodium ascorbate as a reducing agent. After 40 min of incubation at 37 °C, we observed near quantitative product formation. The identity of the coupling product was confirmed by ESI-MS (expected m/z 575.16, found $[M + H]^+$ m/z 576.18).

Palladium-Catalyzed Sonogashira Cross-Coupling of an Alkyne-Functionalized Fluorophore to *p*-Iodophenyl Modified LAP. Having identified the suitable conditions for both the LplA-mediated ligation of *p*-iodophenyl handles to LAP and Sonogashira cross-coupling of alkyne-functionalized fluorescent probes with *p*-iodophenyl compounds, we aimed to combine those individual coupling steps for two-step labeling of LAP (Figure 2). First, LAP was converted with **1b** in LplA-mediated ligation reaction to yield the iodophenyl modified LAP (LAP-**1b**) which was isolated via RP-HPLC in preparative scales and subsequently modified using an alkyne-functionalized fluorescent probe in Sonogashira cross-coupling. Each individual step of the labeling reaction was monitored by HPLC and characterized by mass spectrometry (Figure 2B,C). Product formation of LAP-Fl was observed to be nearly quantitative after 45 min reaction time.

LAP-eDHFR as Model Protein for the Two-Step Labeling Procedure. The general labeling strategy for LAP-eDHFR is outlined in Figure 1. To optimize the Sonogashira cross-coupling for protein labeling, we chose the dihydrofolate reductase from *E. coli* (eDHFR) as model. The labeling experiments were carried out with the fusion protein LAP-eDHFR-6xHis in which eDHFR carries an N-terminal extension containing the lipoic acid acceptor peptide LAP and a C-terminal 6-histidin tag (see Figure S4 in the Supporting Information). As outlined in Figure 3A and B, successful labeling of the model protein can be performed after 40 min of reaction time.

However, thiols can potentially interfere with palladium catalyzed reactions[60−62] and lead to side products over the pathway of thiol−yne coupling. This can be an explanation for the low background signal which we observe for all the negative controls containing the palladium catalyst (Figure 3). In order to investigate the relation between thiols and the palladium catalyst, we used *N*-ethylmaleimide (NEM) to block the cysteine thiols by Michael addition prior to Sonogashira cross coupling. After the blockage of the thiols of LAP-eDHFR and functionalization by $LplA^{W37V}$, Sonogashira cross coupling was subsequently performed. As it can be seen in Supporting Information Figure S7, a 20-fold mass excess of NEM over eDHFR still shows a slight background reaction in the control samples missing the $LplA^{W37V}$, whereas the probes with 50-fold excess do not show any background reactions. These results lead us to the assumption that thiols are involved in the background reactions observed in the negative controls containing the palladium catalyst. This finding might be important for the development of new palladium catalysts for site-specific protein labeling.

CONCLUSION

In this work we presented a two-step labeling approach for peptides and proteins, which is based on the combination of enzyme-mediated labeling utilizing LplA and the Sonogashira cross-coupling reaction. We have shown the attachment of a *p*-iodophenyl derivative to a 13 aa short peptide tag using the $LplA^{W37V}$ mutant with quantitative efficiency even at equimolar substrate concentrations. Further, we demonstrated the

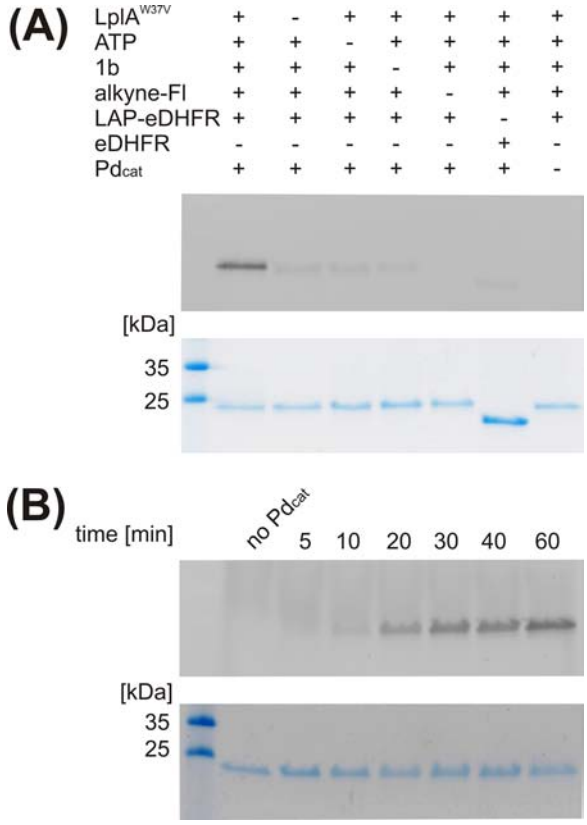

Figure 3. (A) Labeling of functionalized LAP-eDHFR via the Sonogashira cross-coupling. Specific labeling of the LAP-eDHFR fusion protein is performed by using $LplA^{W37V}$ and $Pd(OAc)_2 \cdot [DMADHP]_2$ catalyst. The in-gel fluorescence measurement (upper gel) with the corresponding Coomassie staining (lower gel) is shown. The first lane, containing all necessary reagents for labeling, shows a strong fluorescent band for the efficiently labeled fusion protein. The other lanes represent all negative controls. The presence of a component is indicated by + and the absence by −. (B) Sonogashira cross-coupling of the functionalized LAP-eDHFR with alkyne-Fl and the $Pd(OAc)_2 \cdot [DMADHP]_2$ catalyst reveals saturation for labeling after 30 min.

efficient labeling of the *p*-iodophenyl functionalized peptide with an alkyne-functionalized fluorophore using Sonoashira cross-coupling. For this second step we applied a previously reported palladium-catalyst for application in aqueous media. By applying a bacterial lipoic acid ligase combined with a nontoxic catalyst, this method offers opportunities for bioorthogonal protein labeling. In opposition to other common methods using fluorescent proteins or protein tags like SNAP-, CLIP-, eDHFR-, or Halo-tag, the reduced tag size of the labeling method presented herein minimizes the potential disturbance of a protein's natural function. The advantages of using palladium catalysts and metal complexes under live cell conditions have been discussed in the literature and recent publications,[12,63−65] suggesting an enormous potential of metal complexes that are capable of catalyzing chemical transformations in living organisms.[65,66] The results presented here provide a potential basis for further research in the field of site-specific protein labeling. However, we observed slight background labeling which we assume can be explained by thiolyne-coupling. In order to totally efface this observation, the thiols have to be blocked with NEM. This fact has to be taken into consideration for protein labeling experiments. Further

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improvements have to be done in order to make this method suitable for live cell imaging.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, NMR spectra, and additional figures as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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