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Labeling and Natural Post-Translational Modification of Peptides and Proteins via Chemoselective Pd-Catalyzed Prenylation of Cysteine

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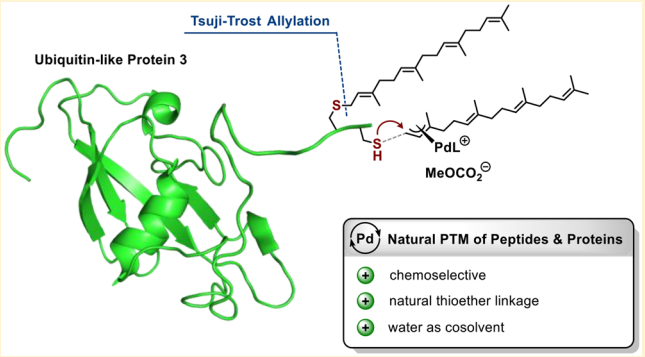
Supporting Information

ABSTRACT: The prenylation of peptides and proteins is an important post-translational modification observed in vivo. We report that the Pd-catalyzed Tsuji−Trost allylation with a Pd/BIPHEPHOS catalyst system allows the allylation of Cys-containing peptides and proteins with complete chemoselectivity and high *n*/*i* regioselectivity. In contrast to recently established methods, which use non-native connections, the Pd-catalyzed prenylation produces the natural *n*-prenylthioether bond. In addition, a variety of biophysical probes such as affinity handles and fluorescent tags can be introduced into Cys-containing peptides and proteins. Furthermore, peptides containing two cysteine residues can be stapled or cyclized using homobifunctional allylic carbonate reagents.



INTRODUCTION

Over the last three decades it has been recognized that post-translational modifications (PTMs) (glycosylation, phosphorylation, sulfation, acylation, lipidation, etc.) play an important role in controlling protein function and localization. Among the PTMs, prenylation is essential for associating certain proteins to specific membranes. A particularly intriguing example for this is the Ras superfamily of small GTPases involved in signal transduction processes that lead to cell growth and differentiation as well as in vesicular trafficking.[1]

For the biophysical and cell biological investigation of proteins in general and of PTMs in particular, chemoselective methods are needed that enable access to modified proteins via synthetic manipulations at the reactive side chains of proteinogenic amino acids using either chemical reagents or a transition-metal catalyst.[2−5] The formation of new covalent bonds allows the attachment of affinity tags, fluorophores, click handles, or PET tracers. Cysteine represents an attractive handle for the introduction of such chemical modifications, as it is the second least frequent amino acid in proteins (1.7%)[6] and shows a very strong inherent nucleophilicity, which makes it especially attractive for reactions with electrophilic reagents. Maleimides[7] and iodoacetamides[8] represent the earliest electrophiles used to alkylate Cys (Figure 1, ***A***) and have been frequently applied to date. More recent developments include a variety of carbonylacrylic reagents as well as vinylpyridines.[9] Since then numerous different bioconjugation strategies have been developed.[2,10] One of them involves the transformation of Cys into dehydroalanine (Dha) upon treatment with 2,5-dibromohexanediamide (DBHDA)[11] or *O*-mesitylenesulfonylhydroxylamine (MSH)[12] (***B***), which is then reacted with a thiol nucleophile (***C***). A disadvantage of this method is that the formation of Dha is associated with racemization at the α-carbon because the diastereoselectivity of the thiol addition in simple Dha peptides is reported to be low.[13] A more direct access to *S*-allylcysteine without epimerization can be accomplished by selenenylsulfide reductive rearrangement (***D***),[14] followed by further derivatization either by olefin cross-metathesis[15] or a Kirmse−Doyle reaction.[16] Alternatively, allylic halides might be used to directly allylate Cys (***E***),[14,17] although these highly reactive reagents are more difficult to handle and preclude more elaborate reagent structures. Very recently Buchwald et al. introduced arylpalladium reagents, which can be used for the arylation of Cys-containing peptides and proteins (***F***).[18] This concept has been extended to Au(III) complexes.[19] Pentelute et al. reported site-selective Cys conjugation with perfluoroarene reagents at the π-clamp motif FCPF (***G***)[20] and ligation with cyclooctynes.[21]

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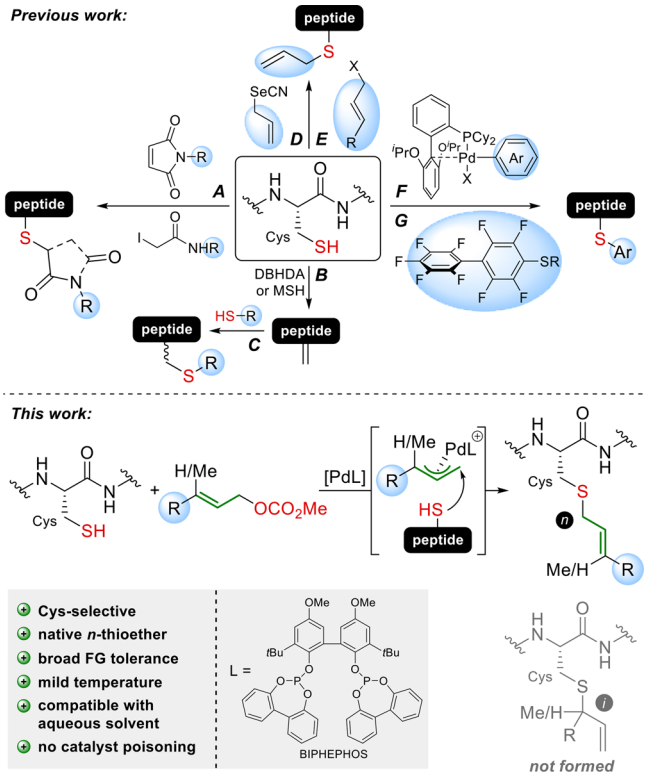

Figure 1. Selected examples of Cys modifications established to date (*A*–*G*) in comparison with the Pd-catalyzed Cys allylation described in this work.

RESULTS AND DISCUSSION

Reaction Optimization. We envisioned that Cys could be selectively modified using the Pd-catalyzed Tsuji–Trost reaction, which would give rise to an allylthioether linkage, as present in naturally prenylated proteins, in a single step. The Tsuji–Trost allylation with *C*-, *N*-, or *O*-nucleophiles is well established in organic synthesis,[22] and Francis et al.[23] have impressively demonstrated the site-selective modification of proteins via Pd-catalyzed *O*-allylation of Tyr residues using the water-soluble phosphine ligand TPPTS. In contrast, the reaction with *S*-nucleophiles has been rarely studied,[24,25] as it faces intrinsic difficulties: (a) *S*-nucleophiles can also function as efficient ligands for Pd and poison the catalyst and (b) thiols are easily oxidized and the reactions have to be carried out under exclusion of air. We hoped that with a prudent choice of ligand we could design a Pd catalyst system suitable for the allylation of Cys-containing peptides and proteins. In contrast to classic allylation procedures[14,17] using an excess of highly reactive allylic halides, a Pd-mediated reaction would allow the use of easily accessible allylic carbonates as electrophiles. These reagents are much more versatile, as they are bench stable and can contain highly functionalized structural motifs. Furthermore, in situ activated electrophiles could be sterically controlled by the Pd complex so that the nucleophilic attack is directed to the terminal end of the η^3-Pd-allyl complex intermediate to produce the *n*-allylation product in high selectivity, as the corresponding *i* product (resulting from internal attack) would be impossible to separate on the peptide or protein level. From a screening of a diverse set of mono- and bidentate phosphorus ligands we identified the bisphosphite ligand BIPHEPHOS as by far the most suitable ligand producing the desired *n* products in high selectivity. Furthermore, the *n*/*i* ratio was found to increase over time even when complete conversion was already reached, indicating the reversibility of this reaction (Table S1).

S-Allylation of Model Substrates. With these optimized conditions in hand, we wanted to apply the Pd-catalyzed *S*-allylation to a dipeptide substrate (**P1**) featuring Tyr as the second amino acid, which could give rise to *O*-allylation as described by Francis et al.[23] before. Importantly, with our Pd/BIPHEPHOS catalyst system we observed exclusively *S*-allylation of Cys, as confirmed via NMR by HMBC experiments (Figure S1). With a series of allylation reagents (Figure 2) we could demonstrate that a diverse set of labeled

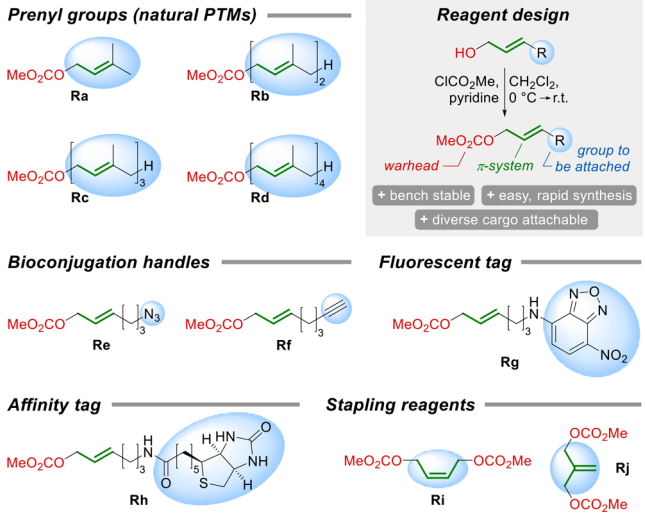

Figure 2. Allylic carbonate reagents prepared for the Pd-catalyzed Cys modification.

peptides could be easily prepared by this method (Table 1, entries 1–7). Moreover, we successfully subjected unprotected glutathione (**P2**) to Pd-catalyzed *S*-prenylation in an aqueous solvent mixture (Table 1, entry 8), indicating a broader applicability of this method for the modification of longer peptides and proteins. This is corroborated by the fact that the reaction proceeds with fast kinetics. Full conversion of 10 mM Ac-Cys-OMe was observed within 10 min upon treatment with 2 equiv of **Ra** in the presence of 2.0 mol % of Pd/BIPHEPHOS at 35 °C. Even 0.5 mol % of the catalyst was found to be sufficient to obtain quantitative conversion after 30 min, demonstrating the high efficiency of the reaction (Figure S2). However, strictly oxygen free conditions were crucial for the activity of the catalyst.

Chemoselective Peptide Modification. As a next step we tested the Pd-catalyzed Cys allylation on a series of more complex oligopeptides. For this purpose, the substrate concentration was reduced to 1 mM to account for the lower solubility of the peptides and the reaction temperature was adjusted to 40 °C to ensure peptide integrity. To compensate for slower kinetics under these conditions, the amounts of Pd and ligand were increased, which fully restored the reactivity of the system. As a relevant target protein for prenylation, we selected ubiquitin-like protein 3 (UBL3) and started out with modifying its *C*-terminal domain (peptide **P3**) with polyprenyl groups, bioconjugation handles, fluorescent and affinity tags, highlighting the versatility of this

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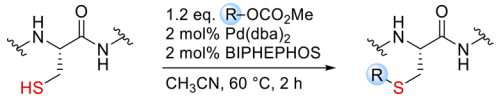
Table 1. Scope of the Pd-Catalyzed Allylation of Small Peptides



Entry	Substrate	R	Product	Yield[a]	*n*/*i*[b]
1	Boc-Cys-Tyr-OMe (**P1**)		**P1a**	88 %	94/6
2	Boc-Cys-Tyr-OMe (**P1**)		**P1b**	84 %	93/7
3	Boc-Cys-Tyr-OMe (**P1**)		**P1c**	75 %	95/5
4	Boc-Cys-Tyr-OMe (**P1**)		**P1e**	82 %	>95/5
5	Boc-Cys-Tyr-OMe (**P1**)		**P1f**	89 %	>95/5
6[c]	Boc-Cys-Tyr-OMe (**P1**)		**P1i**	76 %	>95/5
7[c]	Boc-Cys-Tyr-OMe (**P1**)		**P1j**	70 %	n.a.
8[d]	H-γGlu-Cys-Gly-OH (**P2**)		**P2a**	78 %	>95/5

[a]Isolated yields after column chromatography. [b]Determined by ^{1}H NMR spectroscopy; n.a. = not applicable. [c]0.5 equiv of bifunctional allylation reagent was used. [d]Reaction was performed in 2/1 CH_3CN/H_2O as the solvent for 18 h.

method (Figure 3). Furthermore, we could show that farnesylation is feasible at internal as well as terminal Cys and that this bioconjugation strategy offers access to adjacent and nonadjacent difarnesylated products, which are of special importance in naturally occurring proteins.[26] The high chemoselectivity of this reaction was showcased on a 32aa polypeptide (**P7**) featuring nearly all functional group containing amino acids, which was found to undergo farnesylation exclusively on Cys, as proven by tryptic digest and mass spectrometric analysis (Figure S3).

Peptide Stapling/Cyclization. Having established a series of highly selective monofunctional allylic carbonate reagents that were successfully applied on a broad set of peptide substrates, we were eager to see if our methodology could also be extended to bifunctional allylation reagents. This would enable us to implement an additional type of a peptide stapling protocol,[27] which is based on Pd-mediated *S*-allylation. To this end, we prepared two bifunctional allylic carbonates (**Ri** and **Rj**) with different geometries, which were subjected to Pd-mediated allylation using two α-helical peptides (**P8** and **P9**)[28] with cysteine residues spaced by *i* +3 and *i*+4 as well as peptide **P10** with more distant residues (*i*+11)[29] (Figure 4). Reactions leading to **P8j**, **P9j**, and **P10i**

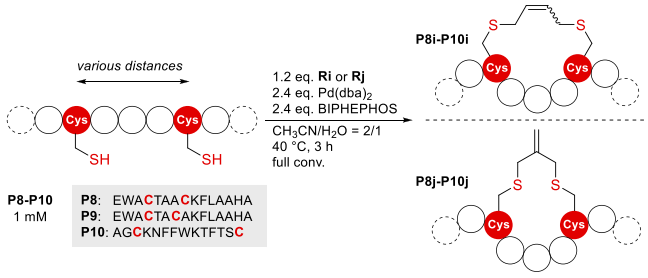


Figure 4. Peptide stapling/cyclization using Pd-mediated allylation. Three model peptides with various distances (*i*+3, *i*+4, *i*+11) between the Cys residues were subjected to stapling/cyclization using two bifunctional reagents with different geometries.

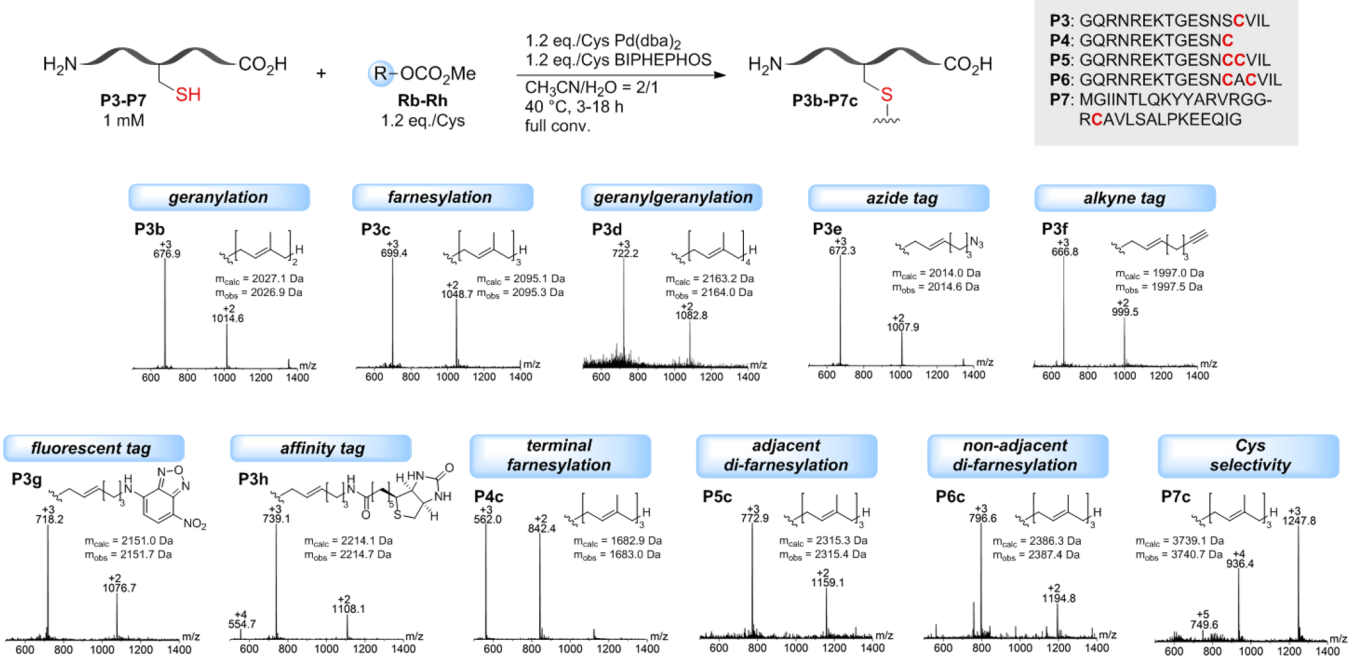


Figure 3. Peptide and reagent scope of the Pd-mediated allylation of oligopeptides. Peptide sequences containing internal, terminal, and multiple Cys residues were subjected to site-selective allylation, enabling the introduction of native prenyl groups and bioconjugation handles (azide/alkyne groups) as well as a fluorescent NBD tag and a biotin affinity tag. All modified peptides were purified, isolated, and characterized by LC-MS analysis.

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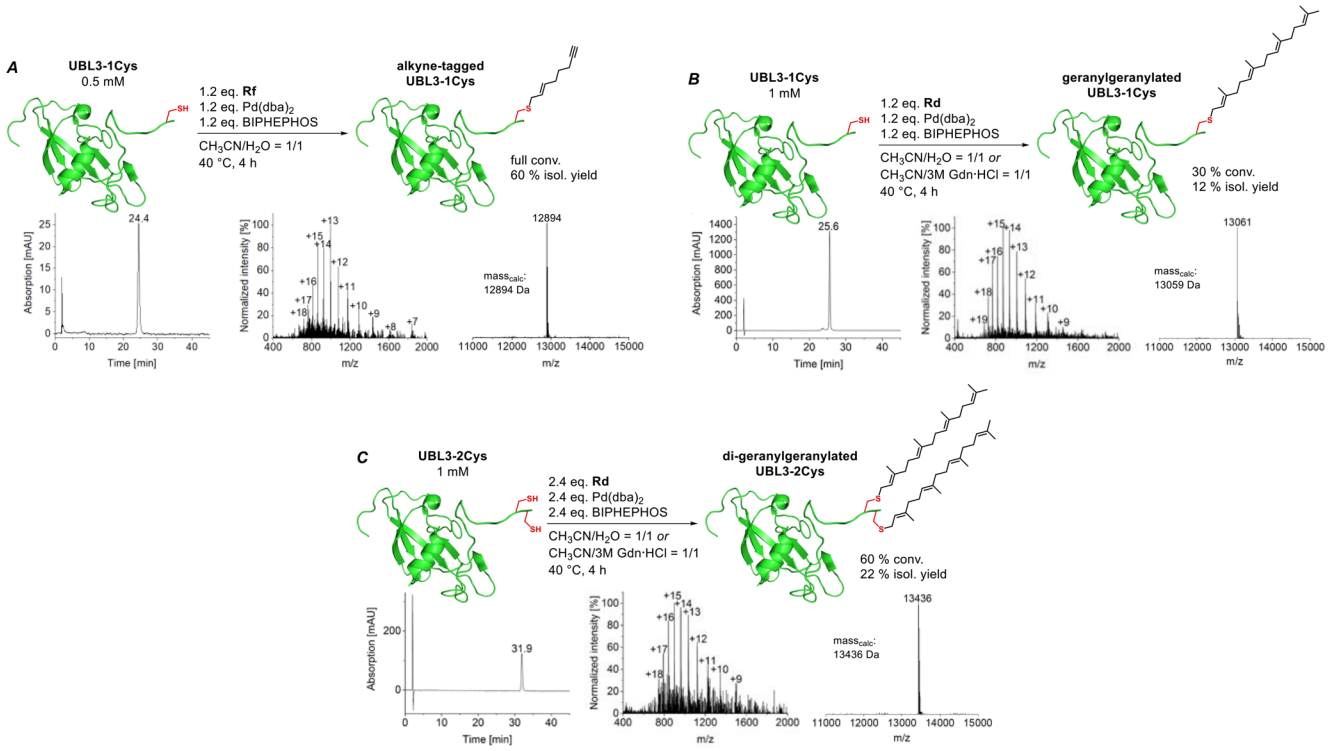

Figure 5. Application of the Pd-mediated allylation for the modification of ubiquitin-like protein 3 (UBL3). Both UBL3 variants with one (A, B) and two (C) *C*-terminal Cys groups were successfully modified when they were treated with 1.2 equiv of allylation reagent per Cys residue. The HPLC traces (214 nm) and mass spectra of the purified products are depicted for UBL3-1Cys-alkyne (A), UBL3-1Cys-Gerger (B), and UBL3-2Cys-$(Gerger)_2$ (C).

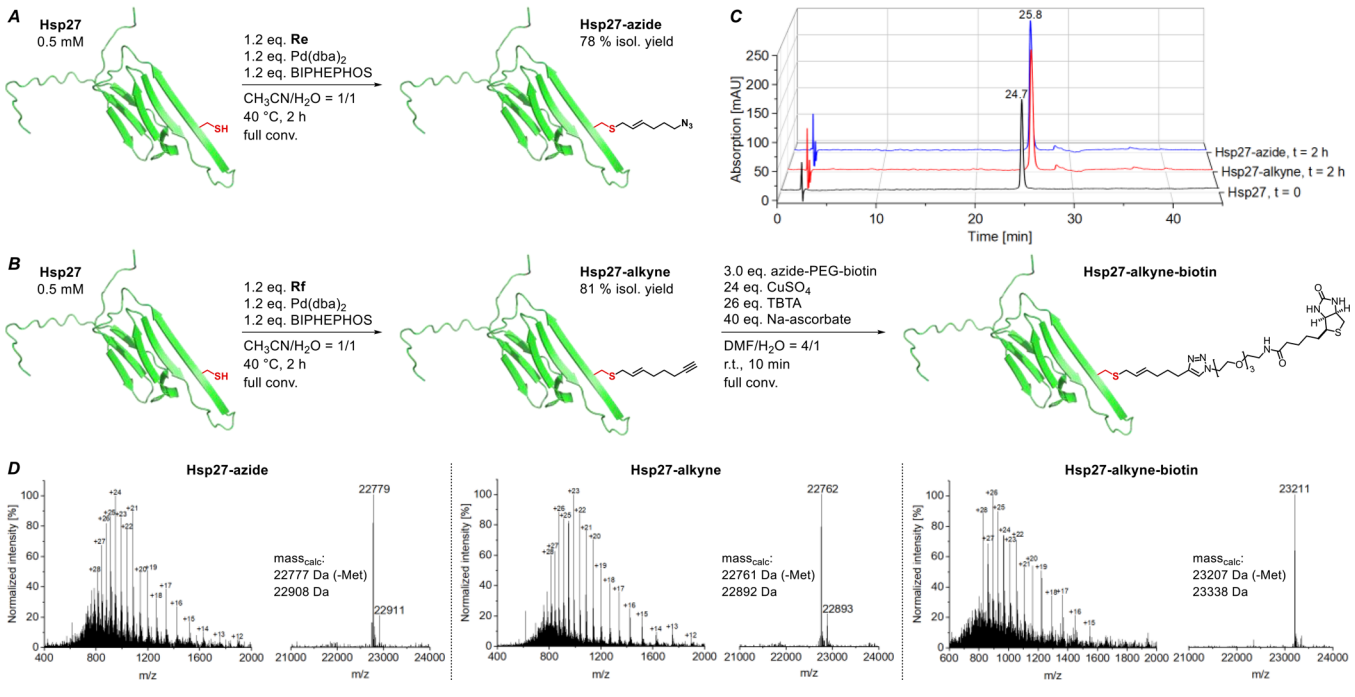

Figure 6. Attachment of azide as well as alkyne handles onto heat shock protein 27 (A, B), which can be employed for click derivatization to introduce labels (biotin). HPLC traces (214 nm) of substrate and crude reaction mixtures (after 2 h) of the Hsp27 modifications (C) and mass spectra of the purified Hsp27 with bioconjugation handles and crude CuAAC product are depicted (D).

gave only one peak corresponding to the desired product, whereas for **P8i** and **P9i** two separate peaks with the expected mass occurred presumably due to the formation of *E*/*Z* isomers. Although the intramolecular reaction was favored for **P10j**, we observed also the dimer of **P10j** (approximately 10%), consisting of two peptides and two staples, as a side product. It is worth mentioning that the stapled products provide motifs for further functionalization by taking

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advantage of the double bond and that allylated peptides with identical staple motifs have recently been shown to function as substrates in decaging strategies using transition-metal catalysis as well.[5]

Modifications of UBL3 Protein. To further evaluate the potential of our method, we chose the full-length protein UBL3 as a substrate, which extends the application beyond classic reactions based on alkyl halides with peptide substrates.[17] UBL3 undergoes post-translational geranylgeranylation in vivo, and direct access to such membrane-bound UBL3 variants will help to elucidate their so far unknown physiological role(s).[30] Two variants, with one and two *C*-terminal Cys groups, were used here since mono- and dilipidation occur in nature. In order to find the appropriate reaction conditions for the Pd-mediated protein allylation, we first applied our alkyne-carrying reagent **Rf** to UBL3-1Cys using a 1/1 CH_3CN/H_2O mixture as the solvent to reconcile protein, reagent, and catalyst solubility. To our delight we observed full conversion in 4 h to the corresponding alkyne-tagged protein that could be isolated in 60% yield with >95% purity after HPLC purification (Figure 5A).

Having demonstrated that our methodology is suitable for the modification of proteins, we introduced the natively occurring geranylgeranyl group with reagent **Rd** into both UBL3 variants using similar conditions. These enabled geranylgeranylation of both UBL3 variants with a conversion of 30% in 4 h. A 1/1 mixture of 3 M aqueous Gdn·HCl with CH_3CN was also tested and increased the conversion of UBL3-2Cys to 60%. After HPLC purification both variants were obtained in high purity (>95%) and with isolated yields of 12% for UBL3-1Cys and 22% for UBL3-2Cys, respectively (Figure 5B,C). Dialysis against a buffer containing 50 mM potassium phosphate at pH 7 gave folded, prenylated UBL3 variants, as confirmed by CD spectroscopy (Figure S4A).

Modifications of Hsp27 Protein. In order to assess more general applications of our Pd-catalyzed protein allylation, we chose heat shock protein 27 (Hsp27) as our next target. It represents a more challenging protein target due to its higher molecular weight and its buried cysteine residue but led to similar prenylation results (Figure S5). Applying reagents **Re** and **Rf**, respectively, under conditions established above for UBL3, gave full conversion into the azide- as well as the alkyne-tagged protein conjugates in just 2 h (Figure 6A,B). The peak-to-peak conversion of Hsp27 is nicely illustrated by HPLC chromatograms at t = 0 and after 2 h (Figure 6C). Both modified Hsp27 variants were isolated in excellent yields (78% and 81%) and high purity (>95%). Direct dissolution of the obtained purified Hsp27 products in 50 mM phosphate buffer at pH 7 led to correctly folded proteins as demonstrated by CD measurements (Figure S4B). To demonstrate the utility of Hsp27-alkyne, we carried out a CuAAC reaction with a commercially available azido-biotin reagent, which led to full conversion into the desired product after only 10 min (Figure 6B).

CONCLUSION

In conclusion, we have developed a chemoselective method for the prenylation, functionalization, and stapling of Cys-containing peptides using Pd/BIPHEPHOS as a catalyst and readily accessible allylcarbonates as reagents. This method was applied to the modification of peptides and proteins for the installation of native prenyl groups as well as artificial bioconjugation handles. In contrast to many established peptide and protein modification reactions, our new Pd-catalyzed Cys-prenylation has the advantage that it forms natural allylthioether linkages as found in prenylated biomolecules and thus can be regarded as a chemical in vitro post-translational modification reaction, which is compatible with all proteinogenic amino acids. In addition, it is general regarding the allylic electrophiles that are applied in minimal excess (1.2 equiv) and therefore provides an efficient tool to introduce labels and tags as well as stabilizing staples into peptides and proteins, affording correctly folded products of high purity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b08279.

Experimental procedures, characterization data, NMR spectra, chromatography traces, mass spectra, and circular dichroism spectra (PDF)

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Notes

The authors declare no competing financial interest.

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