Published in final edited form as:

Chembiochem. 2009 December 14; 10(18): 2934–2943. doi:10.1002/cbic.200900566.

A Minimalist Substrate for Enzymatic Peptide and Protein Conjugation

James W. Wollack^[a], Julie M. Silverman^[b], Christopher J. Petzold^[c], Joseph D. Mougous^[b], and Mark D. Distefano^[a]

^[a]Department of Chemistry University of Minnesota 207 Pleasant Street S.E. Minneapolis, MN 55455 Fax (612) 626-7541

^[b]Department of Microbiology and the Center for Nanotechnology University of Washington Seattle, WA.

^[c]Physical Biosciences Division Lawrence Berkeley National Laboratory Berkeley, CA.

Abstract

Recently a number of non-natural prenyl groups containing alkynes and azides have been developed as handles to perform click chemistry on proteins and peptides ending in the sequence “CAAX”, where C is a cysteine that becomes alkylated, A is an aliphatic amino acid and X is any amino acid. When such molecules are modified, a tag containing a prenyl analog and the “CAAX box” sequence remains. Here we report the synthesis of an alkyne-containing substrate comprised of only nine non-hydrogen atoms. This substrate was synthesized in six steps from 3-methyl-2-buten-1-ol and has been enzymatically incorporated into both proteins and peptides using protein farnesyltransferase. After prenylation the final three amino acids required for enzymatic recognition can be removed using carboxypeptidase Y, leaving a single residue (the cysteine from the “CAAX box”) and the prenyl analog as the only modifications. We also demonstrate that this small tag minimizes the impact of the modification on the solubility of the targeted protein. Hence, this new approach should be useful for applications in which the presence of a large tag hinders the modified protein's solubility, reactivity or utility.

Keywords

alkyne; farnesyltransferase; peptide modification; prenylated peptide; cysteine alkylation

Introduction

Protein prenylation is a post-translational lipid modification characterized by the presence of either a farnesyl group (C15) or a geranylgeranyl group (C20) near the C-terminus of a protein. Prenylated proteins comprise at least 1% of the proteome, are involved in almost all signal transduction pathways, and are overexpressed in 50% of all human tumors.[1] These proteins are either geranylgeranylated or farnesylated depending on the terminal amino acid (X) in the tetrapeptide “CAAX box” sequence that is found at the C-terminus of all prenylated proteins. In this sequence C is a cysteine, A is an aliphatic amino acid and X is any amino acid. Farnesylated proteins are prenylated by PFTase (protein farnesyl transferase) while geranylgeranylated proteins are prenylated by PGGTase (geranylgeranyl transferase). Commonly “CAAX box” proteins that end in A, or M are farnesylated and proteins that end in L are geranylgeranylated.[2] “CAAX” box proteins that end in S can be

either geranylgeranylated or farnesylated. Recombinant yeast[3] and mammalian[4] forms of each of these enzymes have been expressed in *E. coli* and have been used to prenylate both proteins and peptides in ex vivo experiments.

Many studies have been performed on the substrate fidelity of both PGGTase and PFTase. These studies showed that both enzymes can transfer non-natural FPP and GGPP analogs onto “CAAX box” containing substrates. These prenyl-diphosphate analogs have been designed to include fluorescent[5] or photoaffinity[6,7] labels as well as bio-orthogonal tags such as alkynes[8,9] and azides.[10-14] Such prenyl analogs have been transferred to CAAX containing peptides, naturally prenylated proteins, or proteins that have had “CAAX box” sequences appended to their C-terminus through site directed mutagenesis. For instance, Duckworth et al. has tagged GFP containing a C-terminal “CAAX” box with alkyne and azide FPP analogs **1**[9] and **2**[13] (Scheme 1). These analogs were then used to create protein-protein, protein-DNA, and protein-agarose conjugates using Cu(I)-catalyzed click reactions with alkyne labeled proteins, DNA, and agarose.

The aforementioned prenyl analogs are all constructed from geranyl, farnesyl, or geranylgeranyl moieties containing two, three, or four isoprenoid units. In this work, we report the transfer of a non-natural prenyl analog based on a single isoprenoid unit, 3-methyl-2-buten-1-ol, to “CAAX box” proteins and a “CAAX box” peptide using PFTase. This prenyl diphosphate (**3**) is the least bulky alkyne or azide labeled prenyl substrate reported for PFTase to date and thus makes **3** the least intrusive and least hydrophobic modification available for labeling of “CAAX box” containing biomolecules. This is important because modifying molecules with longer hydrophobic chains significantly decreases their solubility. To further extend this minimization approach of modifying biomolecules using the prenyl transfer method, the last three amino acids of the “CAAX box” of a peptide and a protein labeled with alkyne **3** were removed enzymatically. This allows for alkyne labeling of “CAAX box” containing molecules with the net gain of a single cysteine residue attached to a prenyl analog containing only nine non-hydrogen atoms.

Results and Discussion

Previously geranyl diphosphate (GPP) has been shown to be a substrate for PFTase even though it is one isoprenoid unit shorter than the enzyme's natural substrate, FPP.[15] The study reported here concentrates on compound **3**, which is based on dimethylallyl diphosphate (consisting of a single isoprenoid unit), in order to prenylate “CAAX box” containing proteins or peptides. Using **3** as a substrate allows for the selective addition of a clickable alkyne moiety without the addition of a large, hydrophobic isoprenoid.

Synthesis of alkyne diphosphate (**3**)

The synthesis of alkyne-containing prenyl-diphosphate analog **3** was completed in six steps from 3-methyl-2-buten-1-ol (Scheme 2) in a 7.9% overall yield. Dimethylallyl alcohol was protected with dihydropyran, and oxidized to give alcohol **6**. [6,16] The resulting alcohol was converted into alkyne **7** via Williamson ether synthesis by alcohol deprotonation with NaH and subsequent addition of propargyl bromide. Deprotection of **7** with PPTs gave alcohol **8** which was converted to alkyl bromide **9** by treatment with CBr₄ and polymer-supported PPh₃. Bromide **9** was converted to diphosphate **3** by treatment with [(nBu)₄N]₃HP₂O₇[17] and purification using ion-exchange chromatography and RP-HPLC. For subsequent use, compound **3** was dissolved in 25 mM NaHCO₃ and its concentration subsequently quantified using ³¹P-NMR with an internal standard of phosphoric acid. The final product and all intermediates were characterized by ¹H-NMR, ¹³C-NMR, and ESI-MS.

Evaluation of **3** as a substrate for PFTase using a peptide Model

Diphosphate **3** was first investigated as a potential PFTase substrate using a fluorescent HPLC based assay. A “CAAX box” containing peptide, OrG-RTRCVIA-OH (**12**), was prepared by synthesizing the linear peptide sequence by SPPS and coupling the fluorophore (**10**) to the *N*-terminus using DIPCDI, HOBt and DIEA in DMF. Initial attempts to analyze **3**, as well as other longer non-natural-isoprenoid diphosphates, as substrates for PFTase were carried out using dansylated peptides, such as N-Dansyl-GCVIA; however the prenylated products of these reactions were not sufficiently soluble in aqueous media. Thus, we abandoned efforts with the dansylated peptides in favor of Oregon Green-conjugated peptide **12** which was substantially more water soluble due to the presence of multiple charged arginine residues upstream of the required CVIA prenylation recognition sequence. In addition the Oregon Green fluorophore has a much higher quantum yield than the Dansyl fluorophore which in turn required lower peptide concentrations for analysis. In order to determine whether prenyl analog **3** was a substrate for PFTase, it was incubated with the enzyme and peptide **12** at 30 °C in presence of Zn^{2+} and Mg^{2+} . At various time intervals, aliquots were removed and analyzed by HPLC to monitor the conversion from **12** to **13**. Nearly complete conversion (> 85%) was observed after incubating peptide **12** with an excess of **3** for 24 h using typical prenylation conditions. Prenylated product **13** was isolated using HPLC and its identity was confirmed using LC-MS.

Kinetic analysis of **3** using a peptide substrate

While the above experiments established that alkyne **3** was a substrate for PFTase, it was desirable to determine the efficiency of incorporation of **3** compared to the natural substrate, FPP. In previous studies, we have typically employed a continuous spectrofluorometric assay to measure the rate of enzymatic incorporation. That assay monitors the time dependant increase in the fluorescence of a dansylated peptide as it becomes farnesylated; the increase in hydrophobicity due to prenylation results in an increase in the fluorescence intensity of the proximal dansyl group. Unfortunately, the smaller size of **3** decreases the magnitude of this effect; the calculated LogP for farnesol is 5.0 compared to 0.3 for alcohol **8**. Thus no significant change in dansyl group fluorescence was observed when the peptide N-dansyl-GCVIA was incubated with **3** in the presence of PFTase. To circumvent this problem, an HPLC assay based on the separation between peptide substrate **12** and its prenylated congener **13** was employed (using the separation shown in Figure 1). After establishing conditions where the initial rate was linear, reactions containing **12**, **3** (or FPP) and PFTase were performed, fractionated by HPLC and the amount of product formed was calculated by integration of the chromatograms. The apparent $K_{M,app}$ and $V_{max,app}$ parameters were determined for each isoprenoid substrate from direct plots of the rate versus substrate concentration (reported as $K_{M,app}$ and $V_{max,app}$ since the analysis for this bisubstrate reaction was performed at only a single peptide substrate concentration). That analysis gave $K_{M,app}$ values of 2.3 μM and 180 μM for FPP and **3**, respectively, suggesting that **3** binds to PFTase with substantially less affinity (78-fold lower) than FPP. The above kinetic analysis also yielded a value for $V_{max,app}$ for **3** that was 400-fold lower than that for FPP. This was somewhat surprising since the V_{max} value for geranyl diphosphate, an isoprenoid of comparable size to **3**, is similar to that for FPP. Clearly, subtle modifications in the structure beyond the first isoprene unit can still influence the efficiency of incorporation of an isoprenoid substrate. Despite the lower efficiency of incorporation of **3** by PFTase, the ease of synthesis of **3** compared with analogues based on larger isoprenoids coupled with the ready access to significant amounts of PFTase make experiments with this minimized substrate an attractive alternative to larger, more hydrophobic and more complex substrates.

Proteolytic removal of “AAX” residues via carboxypeptidase treatment

After establishing that **3** was a PFTase substrate, we next sought to pursue a minimization strategy that would remove the C-terminal tripeptide (VIA) from the “CAAX box” via proteolysis. If those residues required for PFTase recognition could be removed after prenylation, modification of biomolecules using this method is accomplished with only the net gain of a single cysteine with a modified side chain. Since the “CAAX box” is found at the C-terminus of PFTase substrates, carboxypeptidase Y (CPY) was used to cleave off the ‘AAX’ residues. Previous studies have shown that carboxypeptidase stalls at farnesylated and geranylgeranylated cysteine.[18-20] Hence we predicted that prenylation with **3** followed by proteolysis would result in removal of only the three final C-terminal amino acids, ‘AAX’, that followed the modified cysteine. However, the effect of smaller isoprenylated cysteine residues on carboxypeptidase stalling had not been previously evaluated. Given this uncertainty, we elected to study this question using prenylated peptides prior to proceeding with larger protein substrates where characterization is more difficult.

Accordingly, purified prenylated peptide **13** was converted to its proteolyzed homologue **14** directly or was converted to **14** in one pot by addition of CPY and EDTA after most of **12** was converted to **13** by PFTase. Using LC-MS, the CPY cleavage reaction was observed to be complete within minutes after addition of CPY (used at low micromolar concentrations). Completion was indicated by the absence of any starting material observable by MS and HPLC. CPY-cleaved samples of **13** were also analyzed for cleavage fragments upstream of the modified cysteine side chain. None of the masses for products proteolyzed beyond the modified cysteine were observed. This provides good evidence that CPY cannot cleave through the modified cysteine residue due to its excessive size. Hence, since CPY does not cleave past the modified cysteine, “CAAX box” containing proteins should also have their final three amino acids safely cleaved using this method.

Prenylation and Proteolysis of mCherry

Modifying amino acid residues in proteins is commonly done by reacting Cys or Lys residues with activated succinimidyl esters or maleimides respectively. These protein modification methods while robust, lack the ability to specifically label individual Cys or Lys residues. A plethora of enzymes exist that are responsible for the post-translational modification of proteins.[21] These enzymes require amino acid recognition sequences of varying lengths to guide them to the specific amino acid targeted for modification. In the case of PFTase, the required recognition sequence is only four amino acids long.[22,23] The brevity of this sequence along with the promiscuity of PFTase provides a unique opportunity to use this enzymatic process for the site specific labeling of proteins at cysteine residues near the C-terminus of proteins. This labeling technique can also be used on non-naturally prenylated proteins by appending a CVIA tag at the protein's C-terminus by site directed mutagenesis and subsequently overexpressing the modified protein in *E. coli*. Using this method, virtually any protein can be functionalized with a versatile alkyne moiety.

Having validated the incorporation of **3** into peptides using PFTase, we next sought to apply this to a protein that was not naturally prenylated. PCR based insertion was used to append a CVIA peptide tag to the C-terminus of mCherry to render the fluorescent protein a PFTase substrate.[24] mCherry was used due to its fluorescent properties and relative stability. Previously CVIA-modified mCherry has been prenylated with FPP as well as with non-natural, bioorthogonal prenyl substrates.[24] In this previous work, mCherry was prenylated with an azide-functionalized farnesyl analog and subsequently attached to alkyne-functionalized agarose using Cu(I)-catalyzed, [3+2] Huisgen cycloaddition. In the present work, mCherry-CVIA was prenylated with alkyne-functionalized **3** by PFTase to yield

prenylated protein **16**. The use of isoprenoid diphosphate **3** for this enzymatic transfer is particularly notable since it is the smallest alkyne isoprenoid substrate known for PFTase.

In order to demonstrate the modification of mCherry by **3**, the putatively modified product **16** was subsequently reacted with azide-functionalized agarose using Cu(I)-catalyzed, Huisgen cycloaddition. After reacting for 2 h, the supernatant was removed and the beads were exhaustively washed to remove any non-covalently bound proteins. Fluorescence microscopy revealed that the beads treated with mCherry modified with **3** (**16**) in presence of copper, TBTA ligand, and TCEP turned bright red (Figure 2A) due to the conjugation of the red fluorescent protein via a triazole linkage to the agarose beads. Control experiments where the beads were incubated in the presence of **16** but in absence of copper (Figure 2B) or unprenylated mCherry (Figure 2C) showed no significant fluorescence signal. Thus, these results support the conclusion that mCherry can be modified by PFTase using **3** as a substrate and successfully immobilized.

Reactivity of 'VIA' Deficient mCherry Protein

Prenylated mCherry protein **16** was treated with CPY under conditions that were shown to completely remove the final three amino acids of peptide **13**. After treatment, the protein was concentrated and linked to agarose beads as described above. The fluorescence intensity of beads linked with 'VIA' deficient protein **17** (**20**) was similar to that of beads reacted with full length mCherry protein **16** (**19**) when analyzed using the same imaging conditions (Figure 3). This indicates that the CPY treatment did not proteolyze past the prenylated cysteine within the "CAAX box". If CPY had read through the modified cysteine, then the resulting protein would not have had an alkyne available for cycloaddition to the azide-modified agarose. Also, since the fluorescence intensity of the beads closely matched that of the beads clicked to mCherry protein **16** under the same conditions, the data suggests that the full length and proteolyzed forms of prenylated mCherry (**16** and **17**, respectively) are immobilized with comparable efficiencies; it should be noted that CPY treatment had no deleterious effect on the reactivity or stability of the alkyne modified protein.

Prenylation of Hcp1 with prenyl diphosphate **3**

Compromised solubility in aqueous solutions is a potential problem when modifying proteins with hydrophobic lipid moieties. One motivation for our current study was to define a minimal alkyne-containing PFTase substrate that could circumvent this issue. We chose the homohexameric ring-shaped protein, Hcp1, from *Pseudomonas aeruginosa* as a model for comparing the relative impact of modification with **3** versus larger, previous generation lipid substrates. Hcp1 was chosen for these studies because the C-terminus of the protein readily tolerates fusions, is solvent accessible, and faces the exterior ring face.[25] Furthermore, the feasibility of *in vitro* isoprenoid modification of oligomeric proteins had not yet been ascertained.

We designed and generated an expression construct for Hcp1 containing a C-terminal His₆-tag followed by the "CAAX-box" sequence 'CVIA' (Hcp1-CVIA). This protein was expressed, purified, and modified with **3** or with the larger C15-dihydroazide compound (**2**) in a similar manner described above. Trypsin digestion and subsequent MS analysis of the prenylated samples showed efficient incorporation of the isoprenoid compounds. The predicted C-terminal peptides containing the isoprenoid modification (**3**, m/z = 2132.0; **2**, m/z = 2257.2) were readily detected, however the corresponding unmodified peptide (m/z = 2010.0) was only detected in a control sample containing unreacted Hcp1-CVIA (Figure 4A). Thus, our MS analysis suggests that Hcp1 is nearly quantitatively modified with both substrates under these conditions. Interestingly, our results also indicate that nearly complete derivatization of multimeric proteins can be accomplished.

Next, we evaluated the solubility of Hcp1 modified with **2** or **3**. Semi-quantitative Western blot analysis was used to compare the concentration of protein present in the whole reaction to that which remained in the soluble fraction following high-speed centrifugation. As shown in Figure 4B, soluble C15-modified Hcp1-CVIA (**24**) was not detected, whereas soluble C5-modified Hcp1-CVIA (**22**) was present at a level equivalent to that found in the whole reaction. Thus, the solubility properties of Hcp1-CVIA can be dramatically affected by the nature of the isoprenoid employed for modification.

CPY proteolysis of alkyne-modified Hcp1

Hcp1 that was prenylated with **3** (**22**) was subsequently treated with CPY in order to remove the last three C-terminal amino acids, VIA, that are required for enzymatic prenylation. In a one pot reaction, CPY was added to Hcp1 that was previously incubated with PFTase, **3**, Zn^{2+} , and Mg^{2+} for 2 h. The protein in the reaction mixture was subsequently trypsinized and analyzed by mass spectrometry. The predicted C-terminal peptide containing isoprenoid modification and the loss of the last three amino acids was detected ($m/z = 1848.9$) (Figure 4C); however the prenylated Hcp1 fragment with an intact 'CAAX box' was not found ($m/z = 2132.0$). Thus, prenylation and CPY proteolysis of Hcp1 resulted in the net attachment of a prenylated cysteine residue to the C-terminus of the protein. In end, this allows for the addition of alkyne functionality to a protein in a manner that limits the "tag" present in the final product to a single Cys at the protein's C-terminus.

Conclusion

Modifying "CAAX box" containing proteins with non-natural prenyl diphosphates is an efficient way to append useful functionality near the C-terminus of a polypeptide. Even if a protein does not naturally end in a "CAAX" sequence it can be changed to include the sequence via simple genetic manipulation. Using this method allows virtually any protein to be modified near its C-terminus with bioorthogonal functionality. One potential limitation of this method results from the increase of a protein's hydrophobicity (and hence solubility) after prenyl modification. Using shorter, non-natural prenyl substrates such as compound **3** to modify proteins curtails the increase of hydrophobicity that stems from prenyl modification. Furthermore the final three amino acids of the "CAAX box" that is required for prenylation of proteins and peptides can also be enzymatically removed by treatment with carboxypeptidase Y. The peptidase removes the final three amino acids but does not efficiently read through a prenylated cysteine. Removing these three nonpolar amino acids further reduces the net amount of hydrophobicity needed to be added to modify proteins with this method. In summary, using this approach proteins and peptides can be specifically modified with alkyne functionality with a net gain of a single added cysteine whose side chain is modified with only nine additional non-hydrogen atoms. This should be useful for a variety of applications in protein chemistry.

Experimental Section

General

All synthetic reactions were carried out at rt unless otherwise noted. Thin layer chromatography was done using aluminum plates pre-coated with silica gel 60 from EMD chemicals. Plates were stained using KMnO_4 since all synthesized compounds contained unsaturated functionality. Flash chromatography silica gel (60-200 mesh, 75-250 μM) was obtained from Mallinckrodt Inc. ^1H -NMR spectra were obtained at 200 or 300 MHz. ^{13}C -NMR were obtained at 125 or 75 MHz. ^{31}P -NMR spectra were obtained at 242 MHz. All spectra were obtained on Varian instruments at 25 °C. HPLC separations were performed using a Beckman 166 instrument, equipped with a UV detector, a fluorescence detector

(Chromtech) and a Phenomenex C₁₈ column (Luna, 10 μ M, 10 $\times$ 250 mm) attached to a 5 cm guard column for preparative separations and a Varian C₁₈ column (Microsorb-MV, 5 μ M, 4.6 $\times$ 250 mm) for analytical separations. All UV traces were collected at 214 nm and all fluorescence data was collected using λ_{ex} = 492 nm and λ_{em} = 520 nm. Preparative separations were accomplished using a flow rate of 5 mL/min while analytical runs were carried out using a flow rate of 1 mL/min. Unless otherwise noted solvent A was 0.1% TFA in H₂O and solvent B was 0.1% TFA in CH₃CN. HR-ESI-MS was done using a Bruker Bio TOF II instrument. MALDI-TOF was completed using a 4800 MALDI-TOF/TOF mass analyzer from Applied Biosystems Inc. Recombinant yeast PFTase was overexpressed in *E. coli* BL21(DE3)/pRD577 cells, harvested in 1 L cultures, and purified using ion exchange and affinity chromatography to yield enzyme with a specific activity of 0.22 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. [3] Purified enzyme was stored in 20-100 μ L aliquots in 40% glycerol (v/v) at -80°C. CVIA-modified *m*-cherry was purified using previously developed methods. [24] Concentrations of all proteins and enzymes were determined via Bradford assay using BSA standards. [26] Concentrations of Oregon Green fluorescent peptides were determined spectroscopically (ϵ_{492} = 85,000 M⁻¹·cm⁻¹, 20 mM Tris, pH 9.0). [27] Oregon Green carboxylic acid was purchased from Molecular Probes. ZipTips (μ -C18) were purchased from Millipore. TBTA ligand used for click reactions and diphosphate **2** were synthesized as previously described. [14,28] MS grade trypsin was purchased from Promega. All other chemicals were purchased from Sigma Aldrich.

(E)-2-methyl-4-(tetrahydro-2H-pyran-2-yloxy)but-2-en-1-ol (**7**)

First, previously described procedures were followed to protect the terminal hydroxyl group of 3-methyl-2-buten-1-ol (**4**) with a THP group to provide compound **5**, which was subsequently oxidized using *t*-Bu-OOH and H₂SeO₃ (Scheme 2). [6] The resulting alcohol **6** (0.84 g, 4.5 mmol) was dissolved in 10 mL dry THF and placed into a 38 mL sealed tube with a threaded plug. Next the solution was cooled to 0 °C and NaH (380 mg, 16 mmol) was slowly added to the vessel over 2 min with the observation of some vigorously bubbling, the formation of a white precipitate and a change in color to orange-brown. The tube was capped and slowly allowed to warm to rt. After 1 h, the tube was opened and pre-chilled propargyl bromide (5.5 g, 46 mmol) was added at 0 °C. The tube was resealed and heated to 55 °C for three days at which time the solution was cooled to rt, quenched with H₂O (15 mL), extracted with EtOAc (2 $\times$ 15 mL), washed with H₂O (2 $\times$ 15 mL), concentrated, dried over Na₂SO₄, and purified using flash chromatography (3:1 hexanes/EtOAc, v/v) to yield alkyne **7** as a clear light brown oil (1.0 g, 99% yield). ¹H-NMR (CDCl₃, 300MHz): δ 1.48-1.91 (m, 6H), 1.72 (s, 1H), 2.44 (t, *J* = 2.3 Hz, 1H), 3.49-3.58 (m, 1H), 3.85-3.94 (m, 1H), 4.00 (s, 1H), 4.09 (dd, *J* = 6.6 Hz, 12.3 Hz, 1H), 4.13 (s, 1H), 4.31 (dd, *J* = 6.6 Hz, 12.3 Hz, 1H), 4.65 (t, *J* = 3.0 Hz, 1H), 5.68 (t, *J* = 6.6 Hz, 1H). ¹³C-NMR (75.4 MHz, CDCl₃): δ 14.2, 19.6, 25.5, 30.7, 56.9, 62.3, 63.3, 74.4, 75.1, 79.8, 98.1, 124.8, 135.4. HR-ESI MS for C₁₃H₂₀O₃ [M+Na]⁺, calcd 247.1310, found 247.1293.

(E)-2-(3-methyl-4-(prop-2-ynyloxy)but-2-enyloxy)tetrahydro-2H-pyran (**8**)

PPTs (39 mg, 0.16 mmol) was added to a stirred solution of alkyne **7** (0.49 g, 2.2 mmol) in EtOH (3.0 mL) at 55 °C. The reaction was allowed to proceed for 24 h at reflux, at which time the reaction was concentrated and purified using flash chromatography (3:1 hexanes/EtOAc, v/v) to afford alcohol **8** as a clear light brown oil (220 mg, 71% yield). ¹H NMR (CDCl₃, 300MHz): δ 1.72 (s, 3H), 2.45 (t, *J* = 2.4 Hz, 1H), 3.98 (s, 1H), 4.14 (d, *J* = 2.4 Hz, 2H), 4.24 (d, *J* = 6.7 Hz, 2H), 5.71 (t, *J* = 6.7 Hz, 1H). ¹³C-NMR (75.4 MHz, CDCl₃): δ 14.1, 57.0, 59.1, 74.5, 75.0, 79.8, 127.2, 134.8. HR-ESI MS for C₈H₁₂O₂Na [M+Na]⁺, calcd 163.0730, found 163.0724.

(E)-3-methyl-4-(prop-2-ynloxy)but-2-enyltrihydrogen diphosphate (3)

PPh₃ (polymer-supported beads, 500 mg, 1.9 mmol) was suspended in a solution of alcohol **8** (100 mg, 0.96 mmol) dissolved in dry CH₂Cl₂ (1.0 mL) for 30 min to allow the resin to swell. At that time CBr₄ (380 mg, 1.2 mmol) was added and the reaction was gently stirred for 20 h at which time the beads were removed by filtration and the filtrate was concentrated and purified using flash chromatography (3:1 hexanes/EtOAc, v/v) to afford bromide **9** which was used without additional characterization or purification.

Next Bromide **9** (30 mg, 0.15 mmol) and (*n*-Bu₄N)₃HP₂O₇[14] (340 mg, 0.38 mmol) were dissolved in dry CH₃CN (2.0 mL) and stirred at rt for 3 h. The reaction was then monitored for remaining starting material using TLC (3:1 hexanes/EtOAc, v/v). No starting material was observed. A column was filled with AG 50WX8 ion exchange resin (13 cm × 1.5 cm) which was converted to its ammonium form by adding three column volumes of H₂O/NH₄OH (3:1 v/v) and was equilibrated with buffer C (25 mM NH₄HCO₃/iPrOH, 49:1 v/v) until the pH of the eluant was 7.5-8.0. The reaction mixture was then dissolved in buffer C (20 mL) and applied to the equilibrated column and the eluant collected. Additional buffer C (20 mL) was used to further elute the product and was combined with the preceding fractions. The combined fractions were lyophilized and purified by preparative RP-HPLC (isocratic, solvent A: 25 mM NaHCO₃). R_t = 2.8 min. Product-containing fractions were lyophilized to yield **3** (23 mg, 51% yield). ¹H NMR (D₂O/ND₄OD, 300MHz): δ 1.58 (s, 3H), 2.72 (t, J = 2.5 Hz), 3.91 (s, 2H), 4.04 (t, J = 2.5 Hz, 2H), 4.36-3.44 (m, 2H), 5.48 (t, J = 6.6, 1H). ³¹P NMR (D₂O/ND₄OD, 300MHz): δ -10.14 (d, J = 19.88 Hz, 1P), -8.35 (d, J = 19.9 Hz, 1P). ¹³C-NMR (75.4 MHz): δ 13.7, 56.8, 62.3, 75.1, 76.1, 79.7, 125.2, 136.0. HR-ESI MS for C₈H₁₄O₈P₂ [M-H]⁻, calcd 299.0091, found 299.0064.

OrG-RTRCVIA-OH (12)

The linear peptide sequence NH₂-RTRCVIA-Wang was assembled by automated Fmoc SPPS using Wang resin on an ABI 430 peptide synthesizer. A solution containing DIPCDI (1.9 μL, 12 μmol), Oregon Green carboxylic acid (**10**) (5.0 mg, 12 μmol), HOBt (1.8 mg, 12 μmol), DIEA (3.12 μL, 26 μmol) and DMF (800 μL) was incubated for 10 min at rt and added to NH₂-RTRCVIA-Wang (40 mg, 12 μmol) in a 10 mL solid-phase reaction vessel. The vessel was then tumbled for 14 h in the dark at which time the resin was filtered, washed with DMF (6 × 1 mL) and CH₂Cl₂ (3 × 1 mL) to yield side chain-protected peptide **11** (Scheme 3). The resultant brilliant orange resin was dried in vacuo for 4 h, placed in Reagent K (4 mL, TFA, H₂O, thioanisole, phenol, ethanedithiol / 82.5: 5: 5: 5: 2.5) and tumbled in the dark for 1.5 h. The orange solution containing cleaved peptide was filtered from the resin, dissolved into Et₂O (100 mL), precipitated at -20° C overnight, and centrifuged to form a pellet which was washed with Et₂O (2 × 50 mL). The crude peptide was purified by RP-HPLC (gradient: 0-25% B over 6 min, 25-30% B over 10 min, isocratic at 30% B for 20 min). R_t = 17 min. Product containing fractions were lyophilized to yield **12**. ESI MS for C₅₄H₇₁F₂N₁₃O₁₅S [M+H]⁺, calcd 1212.5, found 1212.5.

Prenylation of OrG-RTRCVIA with alkyne 3

An enzymatic reaction (1 mL) containing 2.4 μM **12**, 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 10 mM MgCl₂, 100 μM ZnCl₂, 44 μM **3**, and 94 μL of 13 μM PFTase was performed in a 30 °C water bath. To ensure complete reduction of any disulfide present in the starting peptide all reagents except **3** and enzyme were incubated at rt for 1 h. Following addition of **3**, reactions were initiated by addition of enzyme. At various times aliquots (50-100 μL) were removed and analyzed by analytical HPLC (gradient: 0-60% B over 60 min). The reaction was deemed complete after 24 h and was purified using the above HPLC column and method. The starting material (**12**) had a R_t of 38.2 min and the prenylated product (**13**)

had a R_t of 44.2 min (Figure 1). ESI MS for $C_{62}H_{81}F_2N_{13}O_{16}S$ $[M+H]^+$, calcd 1334.6, found 1334.6.

Kinetic analysis of **3** using a peptide substrate

Enzymatic PFTase prenylation rates using **3** were determined using an HPLC based assay. An assay solution containing 60 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 12 μM $ZnCl_2$, 18 mM DTT, and 2.9 μM **12** was prepared and allowed to incubate at rt for 45 min in order to ensure complete reduction of any disulfides. In 1.5 mL centrifuge tubes, 50 μL solutions of **3** were prepared so when they were diluted into 250 μL of assay solution the final concentration of **3** would be 1.0, 2.0, 5.0, 10, 15, 30, 50 or 200 μM . After the alkyne containing solutions were diluted into assay buffer, PFTase (30 μL , 1.38 mg/mL) was added to each centrifuge tube and the reaction was incubated for 90 min at 30 °C at which time each reaction was flash frozen. Each reaction was analyzed using an HPLC equipped with a fluorescence detector using the separation conditions specified above for prenylation of OrG-RTRCVIA. Fluorescent peaks corresponding to the starting material and product were integrated and used to calculate the concentration of prenylated product in solution. Three different samples were prepared for each concentration and the average concentration of product from the three reactions was used to calculate $K_{M,app}$ and $V_{max,app}$ for **3**.

A similar procedure was performed using 0.1, 0.3, 0.5, 1.0, 3.0, 18, and 36 μM concentrations of FPP. For FPP, PFTase (30 μL , 0.046 mg/mL) was used to initiate the reaction and the reaction was ran for 5 min at 30 °C before being flash frozen in liquid nitrogen.

Proteolysis of prenylated peptide **13** with carboxypeptidase

A prenylation reaction (6.5 mL) containing 2.4 μM **12**, 50 mM Tris-HCl, pH 7.5, 15 mM DTT, 10 mM $MgCl_2$, 100 μM $ZnCl_2$, 44 μM **3**, and 94 μL of 13 μM PFTase was incubated for 4.5 h at 30 °C and was determined greater than 60% complete by RP-HPLC. To ensure complete reduction of any disulfide present in the starting peptide all reagents except **3** and enzyme were incubated at rt for 1 h and the reaction was initiated by the addition of these two reagents as noted above. The prenylation reaction was allowed to incubate for 2 h at which time CPY (50 μg) dissolved in 20 mM Tris pH 8.0 containing 20 mM EDTA (1.0 mL) was added to the reaction. The reaction was removed from the 30 °C water bath and placed at rt for 2 h at which time the proteolyzed product (**14**) was purified by preparative RP-HPLC (gradient: 0-60% B over 60 min). The product containing fractions were concentrated to remove CH_3CN and re-purified using analytical HPLC (gradient: 0-60% B over 60 min) to obtain a product of greater than 98% purity, R_t = 40.2 min (Figure 1). ESI MS for $C_{48}H_{56}F_2N_{10}O_{13}S$ $[M+H]^+$, calcd 1051.4, found 1051.4.

Prenylation of mCherry-CVIA with **3**

An enzymatic reaction (10 mL) was prepared that contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 15.0 mM DTT, 2.4 μM mCherry-CVIA (**15**), 40 μM **3**, and PFTase (300 μL). In order to reduce any disulfide linkages, all reagents except **3** and enzyme were premixed and incubated together for 1 h at rt. Following addition of **3** and PFTase the reaction was incubated at 30 °C for 4 h. At this time the reaction was concentrated to 500 μL using a Centricon Ultra YM-10 filtration device and purified from excess alkyne **3** using a NAP-5 column. The concentration of the prenylated product (**16**) was determined spectroscopically ($\epsilon_{492}=72,000 M^{-1}cm^{-1}$).[29]

Carboxypeptidase Y proteolysis of alkyne modified mCherry

Carboxypeptidase Y (75 μ g) dissolved in 20 mM Tris-HCl, pH 7.5 (20 μ L), was added to prenylated protein **16** (90 μ L, 165 μ M) followed by the addition of 20 mM Tris-HCl buffer, pH 8.0 that was supplemented with 20 mM EDTA (60 μ L). The reaction was incubated at rt for 3 h, followed by incubation at 4 °C overnight to yield truncated mCherry protein **17** (Scheme 4). The next day the reaction was concentrated to approximately 60 μ L using a Microcon YM-10 centrifugal device and quantified by a Bradford assay using BSA standards.[26]

Immobilization of Alkyne Modified mCherry to Azide Functionalized Agarose

Two samples containing azide modified agarose (**18**) (100 μ L) were washed three times with 50 mM NaH₂PO₄, pH 7.3. One of the samples was suspended in 50 mM NaH₂PO₄, pH 7.3, containing 80 μ M alkyne modified mCherry (**16**), 100 μ M TBTA ligand, 1 mM CuSO₄, and 1 mM TCEP (60 μ L). The other sample was suspended in the same mixture but without CuSO₄. The samples were reacted for 4 h at rt at which time the supernatant was collected and the beads were washed with 50 mM NaH₂PO₄ that contained 1M NaCl (3 $\times$ 100 μ L). The copper deficient sample contained beads that looked the same before the reaction (white in color); however the reaction that included copper contained beads that were visibly red without the use of UV light. Under fluorescent light from an inverted microscope fitted with a Cy 5 filter, the beads that were treated with copper were bright red while the copper deficient control showed no fluorescence (Figure 2, compare panels A and B).

Control reactions done in the absence of TBTA and TCEP did not indicate conjugation of alkyne modified mCherry (**16**) to azide functionalized beads (data not shown). Control reactions performed using unmodified mCherry protein **15** were also unreactive (Figure 2, Panel C). The same procedure was used with prenylated mCherry protein that was treated with CPY (**17**) and yielded identical results (Compare Figure 2A to 3A).

Hcp1-CVIA cloning, over-expression and purification

The CVIA-tag was appended to the C-terminus of Hcp1 pET29B_*hcp1* [25] by standard cloning procedures with PCR forward primer 5'–GGAGATATACATATGGCTGTTGATATGTTTCATCAAG–3' and reverse primer 5'–GGATCCTCAGGCGATCACGCAGTGGTGGTGGTGGTGGTGGTCTCG–3'. This places the amino acid sequence CVIA C-terminal to the 6x His-tag. Hcp1-CVIA was expressed and purified as described previously.[25]

Prenylation and CPY proteolysis of Hcp1 with **3**

An enzymatic reaction (0.3 mL) was prepared that contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 μ M ZnCl₂, 15 mM DTT, 2.4 μ M Hcp1 (**21**), 57 μ M **3**, and PFTase (50 μ L). In order to reduce any disulfide linkages, all reagents except **3** and enzyme were premixed and incubated together for 1 h at rt. Following addition of **3** and PFTase, the reaction was incubated at 30 °C for 3 h, at which time an aliquot was removed for analysis by mass spectrometry. MALDI-MS for C-terminal fragment QNVQALEHHHHHHC[C₈H₁₁O]VIA (the expected tryptic peptide containing the desired alkyne modification), C₉₄H₁₃₈, N₃₂, O₂₄, S, [M+H]⁺, calcd 2132.0, found 2132.0 (Figure 4A).

For CPY treated samples, a prenylation reaction was performed as described above (Scheme 5). After 2 h, CPY (100 μ g) dissolved in 20 mM Tris, pH 7.5 (40 μ L) was added to the reaction and allowed to react at rt without stirring. After 3 h, the reaction was stored at 4 °C until further analysis by mass spectrometry. MALDI-MS for C-terminal fragment QNVQALEHHHHHHC[C₈H₁₁O], C₈₀H₁₁₃, N₂₉, O₂₁, S, [M+H]⁺, calcd 1848.8, found 1848.8 (Figure 4C).

Prenylation of Hcp1 with 2

An enzymatic reaction (3.1 mL) was prepared that contained 50 mM Tris·HCl, pH 7.5, 10 mM MgCl₂, 10 μM ZnCl₂, 15 mM DTT, 2.4 μM Hcp1 (**21**), 20 μM **2**, and PFTase (50 μL). Thiol reduction was performed as described for the prenylation of Hcp1 with **3**. Following addition of **2** and enzyme, the reaction was incubated at 30 °C for 3 h and subsequently analyzed by mass spectrometry. MALDI-MS for C-terminal fragment QNVQALEHHHHHCVIA[C₁₅H₂₆N₃], C₁₀₁H₁₅₃, N₃₅, O₂₃, S, [M+H]⁺, calcd 2257.2, found 2257.2 (Figure 4A).

Tryptic digest of prenylated Hcp1-CVIA and prenylated Hcp1-CVIA treated with carboxypeptidase

Prenylated (**22**) and unprenylated (**21**) Hcp1-CVIA were digested in PFTase reaction buffer using MS grade trypsin at 1:20 (w/w trypsin to Hcp1) for 18 h at rt. The reaction was stopped with 5% acetic acid and peptides were purified according to the manufacturer's protocol using μ-C18 ZipTips.

Mass spectrometry of tryptic peptides

Peptide samples (0.4 μL) were spotted onto a target plate along with 0.6 μL matrix solution (α-cyanohydroxycinnamic acid in 0.1% TFA, 70% CH₃CN) and allowed to air-dry. Analysis was accomplished on a 4800 MALDI-TOF/TOF mass analyzer operating in the positive mode. Each MS spectrum is the accumulated spectra of 1000 laser shots (Nd:YAG (355 nm), 200 Hz) consisting of 50 shots/sub-spectrum and 20 sub-spectra over a mass range of 800 - 4000 Da. The first five shots were discarded and the accelerating voltage was operated at 20 kV. To each spot, 20 fmol of Glu-fibrinopeptide D were added to serve as an internal calibration mass.

Prenylated Hcp1-CVIA solubility assay

To determine the relative solubility of Hcp1-CVIA conjugated to either **2** or **3**, an aliquot of the final reaction mixture was removed and subjected to centrifugation at 16,000 × g at 4 °C for 30 min. Equal volumes of the supernatant of this sample and untreated, homogenized reaction mixture were analyzed for the presence of Hcp1 using Western blotting procedures described previously (Figure 4B).[30]

Acknowledgments

The authors thank Dr. Daniel Mullen for guidance on peptide synthesis, Gregg Amundson for assistance in microscopic imaging, Stephan Lenevich for determining the concentration of prenyl diphosphates using ³¹P-NMR, Brock Matter, Rebecca Guza, Sean Murray, and Dr. Peter Villalta for assistance in obtaining ESI-MS data, and Letitia Yao for assistance in NMR. Portions of the mass spectrometry done in this work was completed at the University of Minnesota Masonic Cancer Center, a comprehensive cancer center designated by the National Cancer Institute supported in part by P30CA77598. This work was supported by the National Institutes of Health Grant No GM058842, CA104609, and T32GM008347.

References

1. Barbacid M. Annu. Rev. Biochem. 1987; 56:779. [PubMed: 3304147]
2. Cox AD. Methods Enzymol. 1995; 250:105. [PubMed: 7651143]
3. Mayer MP, Prestwich GD, Dolence JM, Bond PD, Wu H, Poulter CD. Gene. 1993; 132:41. [PubMed: 8406041]
4. Omer CA, Kral AM, Diehl RE, Prendergast GC, Powers S, Allen CM, Gibbs JB, Kohl NE. Biochemistry. 1993; 32:5167. [PubMed: 8494894]
5. Owen DJ, Alexandrov K, Rostkova E, Scheidig AJ, Goody RS, Waldmann H. Angew. Chem. 1999; 111:570. Angew. Chem. Int. Ed. Engl. 1999; 38:509.

6. Turek TC, Gaon I, Distefano MD, Strickland CL. *J. Org. Chem.* 2001; 66:3253. [PubMed: 11348105]
7. Chehade KAH, Kiegiel K, Isaacs RJ, Pickett JS, Bowers KE, Fierke CA, Andres DA, Spielmann HP. *J. Am. Chem. Soc.* 2002; 124:8206. [PubMed: 12105898]
8. Hosokawa A, Wollack JW, Zhang Z, Chen L, Barany G, Distefano MD. *Int. J. Pept. Res. Ther.* 2007; 13:345.
9. Duckworth BP, Zhang Z, Hosokawa A, Distefano MD. *ChemBioChem.* 2007; 8:98. [PubMed: 17133644]
10. Xu J, DeGraw AJ, Duckworth BP, Lenevich S, Tann C-M, Jenson EC, Gruber SJ, Barany G, Distefano MD. *Chem. Biol. Drug Des.* 2006; 68:85. [PubMed: 16999773]
11. Nguyen UTT, Cramer J, Gomis J, Reents R, Gutierrez-Rodriguez M, Goody RS, Alexandrov K, Waldmann H. *ChemBioChem.* 2007; 8:408. [PubMed: 17279592]
12. Labadie GR, Viswanathan R, Poulter CD. *J. Org. Chem.* 2007; 72:9291. [PubMed: 17979291]
13. Duckworth BP, Xu J, Taton TA, Guo A, Distefano MD. *Bioconjugate Chem.* 2006; 17:967.
14. Duckworth BP, Chen Y, Wollack JW, Sham Y, Mueller JD, Taton TA, Distefano MD. *Angew. Chem.* 2007; 119:8795. *Angew. Chem., Int. Ed. Engl.* 2007; 46:8819. [PubMed: 17935099]
15. Pompliano DL, Schaber MD, Mosser SD, Omer CA, Shafer JA, Gibbs JB. *Biochemistry.* 1993; 32:8341. [PubMed: 8347630]
16. Turek TC, Gaon I, Distefano MD. *Tetrahedron Lett.* 1996; 37:4845.
17. Davisson VJ, Woodside AB, Neal TR, Stremler KE, Muehlbacher M, Poulter CD. *J. Org. Chem.* 1986; 51:4768.
18. Clausen VA, Edelstein RL, Distefano MD. *Biochemistry.* 2001; 40:3920. [PubMed: 11300771]
19. Edelstein RL, Weller VA, Distefano MD, Tung JS. *J. Org. Chem.* 1998; 63:5298.
20. Ashby MN, Rine J. *Methods Enzymol.* 1995; 250:235. [PubMed: 7651155]
21. Chow M, Der CJ, Buss JE. *Curr. Opin. Cell Biol.* 1992; 4:629. [PubMed: 1419045]
22. Schafer WR, Rine J. *Annu. Rev. Genet.* 1992; 26:209. [PubMed: 1482112]
23. Zhang FL, Casey PJ. *Annu. Rev. Biochem.* 1996; 65:241. [PubMed: 8811180]
24. Chung JA, Wollack JW, Hovlid ML, Okesli A, Chen Y, Mueller JD, Distefano MD, Taton TA. *Anal. Biochem.* 2009; 386:1. [PubMed: 18834849]
25. Ballister ER, Lai AH, Zuckermann RN, Cheng Y, Mougous JD. *Proc. Natl. Acad. Sci.* 2008; 105:3733. [PubMed: 18310321]
26. Bradford MM. *Anal. Biochem.* 1976; 72:248. [PubMed: 942051]
27. Haugland, R.; P.. *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies.* 10th Edition. 2005.
28. Chan TR, Hilgraf R, Sharpless KB, Fokin VV. *Org. Lett.* 2004; 6:2853. [PubMed: 15330631]
29. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. *Nat. Biotechnol.* 2004; 22:1567. [PubMed: 15558047]
30. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordonez CL, Lory S, Walz T, Joachimiak A, Mekalanos JJ. *Science.* 2006; 312:1526. [PubMed: 16763151]

Scheme 1.

Prenylation reaction catalyzed by PFTase and examples of several non-natural bioorthogonal PFTase substrates.

Scheme 2.

Synthesis of a minimal alkyne containing substrate (**3**) for PFTase.

Scheme 3.
Synthesis and subsequent prenylation and 'VIA' proteolysis of OrG-RTRCVIA.

Scheme 4.

Prenylation, 'VIA' proteolysis, and immobilization of mCherry-CVIA using (3), CPY, and azide modified agarose.

Scheme 5.

Prenylation of Hcp1 with prenyl alkyne **3** and prenyl azide **2**. Hcp1 prenylated with alkyne **3b** was subsequently proteolyzed using CPY.

Figure 1.

Reverse-phase HPLC analysis of purified peptides **12**, **13**, and **14**. Chromatogram (1): PFTase peptide substrate OrG-RTRCVIA (**12**) ($t_R = 35$). Chromatogram (2): prenyl alkyne modified OrG-RTRCVIA (**13**) ($t_R = 41$). Chromatogram (3): Prenylated peptide **14** ($t_R = 36$) that has had the last three amino acids of its “CAAX box” removed by the enzyme CPY. Fluorescence intensity is reported in relative fluorescence units (RFU).

Figure 2.

A) Reaction of azide-functionalized agarose with alkyne labeled mCherry (**16**) in the presence of 1 mM CuSO_4 . B) Reaction of azide-functionalized agarose with alkyne labeled mCherry (**16**) in the absence of CuSO_4 . C) Reaction of mCherry-CVIA (**15**) in presence of 1 mM CuSO_4 . All reactions were run in the presence of 80 μM protein, 1 mM TCEP, 100 μM TBTA ligand, and 50 mM NaH_2PO_4 , pH 7.3. Bright field images are on left and fluorescent microscope images are on right. Bars in lower right corners represent 200 μM .

Figure 3.

A) Reaction of azide-functionalized agarose with alkyne labeled 'VIA' deficient mCherry (**17**) in the presence of 1 mM CuSO₄. B) Reaction of azide-functionalized agarose with alkyne labeled 'VIA' deficient mCherry (**17**) in the absence of 1 mM CuSO₄. All reactions were ran in the presence of 80 μ M protein, 1 mM TCEP, 100 μ M TBTA ligand, and 50 mM NaH₂PO₄, pH 7.3. Bright field images are on left and fluorescent microscope images are on right. Bars in lower right corners represent 200 μ M.

Figure 4.

P. aeruginosa Hcp1 is efficiently prenylated by PFTase and its solubility depends on the length of the lipid moiety. **A)** MALDI mass spectra of tryptic digests of unmodified Hcp1-CVIA and Hcp1-CVIA modified with either **2** (**24**) or **3** (**22**). An asterisk in each spectrum indicates the position of the diagnostic peptide. A zoomed area surrounding this region is provided in the insets. The spectra lack great overall similarity due to the presence or absence of additional peaks contributed by PFTase tryptic peptides. In the case of Hcp1-CVIA, reactions with **3**, excess PFTase was utilized; therefore, PFTase-originating peak are particularly prominent. **B)** C5-Alkyne modified Hcp1 (**22**) displays improved solubility properties relative to C15-Azide modified Hcp1. Western blot analysis of whole reaction (R) and soluble (S) fractions of Hcp1-C15-Azide (C15, **24**) and Hcp1-C5-Alkyne (C5, **22**). **C)** MALDI mass spectra of tryptic digests of (**22**) before (top) and after (bottom) treatment with carboxypeptidase (CPY). The arrows indicate the position of the cleaved peptide.