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Simultaneous Site-Specific Dual Protein Labeling Using Protein Prenyltransferases

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Abstract

Site-specific protein labeling is an important technique in protein chemistry and is used for diverse applications ranging from creating protein conjugates to protein immobilization. Enzymatic reactions, including protein prenylation, have been widely exploited as methods to accomplish site-specific labeling. Enzymatic prenylation is catalyzed by prenyltransferases, including protein farnesyltransferase (PFTase) and geranylgeranyltransferase type I (GGTase-I), both of which recognize C-terminal CaaX motifs with different specificities and transfer prenyl groups from isoprenoid diphosphates to their respective target proteins. A number of isoprenoid analogues containing bioorthogonal functional groups have been used to label proteins of interest via PFTase-catalyzed reaction. In this study, we sought to expand the scope of prenyltransferase-mediated protein labeling by exploring the utility of rat GGTase-I (rGGTase-I). First, the isoprenoid specificity of rGGTase-I was evaluated by screening eight different analogues and it was found that those with bulky moieties and longer backbone length were recognized by rGGTase-I more efficiently. Taking advantage of the different substrate specificities of rat PFTase (rPFTase) and rGGTase-I, we then developed a simultaneous dual labeling method to selectively label two different proteins by using isoprenoid analogue and CaaX substrate pairs that were specific to only one of the prenyltransferases. Using two model proteins, green fluorescent protein with a C-terminal CVLL sequence (GFP-CVLL) and red fluorescent protein with a C-terminal CVIA sequence (RFP-CVIA), we demonstrated that when incubated together with both prenyltransferases and the selected isoprenoid analogues, GFP-CVLL was specifically modified with a ketone-functionalized analogue by rGGTase-I and RFP-CVIA was selectively labeled with

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Supporting Information Available: Descriptions of the syntheses of analogues **2** and **8**, DNA sequence for GFP-CVIA (**10a**), DNA sequence for RFP-CVIA (**13a**), kinetic study of analogue **2** with yPFTase, Scheme and MS results of enzymatic incorporation of analogue **2** into GFP-CVIA (**S13**) and GIP-CVIM (**S15**), summary of the results from initial fluorescence assays with isoprenoid analogues as possible substrates for rGGTase-I, MS results for rPFTase reaction with GFP-CVIA (**10a**) and **5**, HPLC results for rPFTase prenylation reactions, MS results for GFP-CVIA (**10a**) labeling using the dual labeling system in the presence of both **5** and **8**, SDS-PAGE results for a comparison of boiled and unboiled protein samples, Scheme and MS results for simultaneous GFP-CVLL (**9a**) and RFP-CVIA (**13a**) labeling with analogue **5** and **8**, and SDS-PAGE analysis of GFP-RFP dimer. This material is available free of charge via the Internet at <http://pubs.acs.org>.

an alkyne-containing analogue by rPFTase. By switching the ketone-containing analogue to an azide-containing analogue, it was possible to create protein tail-to-tail dimers in a one-pot procedure through the copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction. Overall, with the flexibility of using different isoprenoid analogues, this system greatly extends the utility of protein labeling using prenyltransferases.

Graphical Abstract

Keywords

Site-specific protein labeling; Simultaneous protein labeling; Protein dimerization; Protein farnesyltransferase; Protein geranylgeranyltransferase type I; Isoprenoid analogues

Introduction

Compared to conventional non-specific modifications of proteins, site-specific protein labeling is becoming increasingly utilized. This labeling method produces homogeneously modified proteins, which can be used in many different applications in chemical biology and biotechnology.^{1,2} Site-specific labeling is particularly helpful in creating chimeric molecules including protein-protein conjugates,^{3,4} protein-polymer conjugates^{5–8} and protein-small molecule conjugates^{9,10} with uniform composition, which is highly desirable for many biomedical applications.¹¹ Moreover, with site-specifically labeled proteins, oriented protein immobilization with uniform surface coverage can also be achieved which is valuable for the development of protein microarrays and for the surface immobilization of enzymes in industrial applications.^{12–14}

Enzymatic protein labeling has been widely employed to produce site-specifically labeled proteins.^{15,16} In this method, a protein of interest is modified with a genetically encoded tag that is recognized by an enzyme, which then transfers its substrate (usually modified with bioorthogonal functional groups) to the tag sequence encoded within the protein. This type of site-specific labeling is widely used due to the fact that it is carried out under mild conditions, can be driven to high levels of conversion and can be used to install a range of different functional groups, allowing a variety of applications.¹⁷

Protein prenylation, an enzyme-catalyzed post-translational modification, has been exploited as a labeling technique because of its high specificity and its small recognition sequence

(four C-terminal residues) on the protein substrate.^{18–20} In the prenylation reaction, an isoprenoid group is attached to a cysteine residue near the C-terminus of target proteins (Figure 1). This reaction is catalyzed by protein prenyltransferases, including protein farnesyltransferase (PFTase) and geranylgeranyltransferase type I (GGTase-I). Both of these enzymes recognize a C-terminal CaaX motif, where the “C” represents a cysteine residue, “a” represents an aliphatic amino acid and the “X” residue determines whether the protein will be recognized by PFTase and/or GGTase-I.^{21,22} These two enzymes also have a different preference regarding the isoprenoid substrates they accept, where PFTase naturally accepts farnesyl diphosphate (FPP), while GGTase-I generally utilizes geranylgeranyl diphosphate (GGPP) due to its larger isoprenoid-binding site.^{23,24}

In order to site-specifically label a protein bearing a CaaX motif, several isoprenoid analogues containing different bioorthogonal functional groups, including alkynes,^{25,26} different aldehydes^{18,27} and azides^{26,28} have been reported for applications ranging from the synthesis of protein conjugates to probing the prenylome. Substrates for PFTase have been used for site-specific protein labeling to create PEGylated glucose-dependent insulinotropic polypeptide (GIP) as a potential type 2 diabetes treatment by using an aldehyde-containing analogue¹⁸ and to build antibody arrays for multiple antigen detection with an alkyne-containing analogue.¹⁹ A substrate for GGTase-I has been employed to detect and study geranylgeranylated proteins in both cultivated cells and mammalian tissue samples.²⁹

While site-specific labeling of one protein is highly useful for a variety of applications and has been explored extensively, the ability to simultaneously label two proteins is also quite useful but has been studied in only a limited number of cases. In one strategy, fusion proteins with intrinsically fluorescent proteins have been used to label multiple protein targets inside cells for visualization and detection.³⁰ In a different approach, a combination of SNAP and CLIP tagging was used to label two protein targets with fluorophores simultaneously and selectively inside cells.³¹ The insertion of alkyne and azide containing non-natural amino acids into two different proteins has also been utilized to simultaneously label these proteins in fixed cells.³² Glycans, modified with different bioorthogonal functional groups, have been used for metabolic labeling of different cell surface glycoproteins for two color imaging.^{33,34} However, these methods also manifest various limitations. For example protein fusions with intrinsic fluorescent proteins (eg GFP) or large tags (SNAP and CLIP) may interfere with protein function while the introduction of non-natural amino acids may suffer from low incorporation efficiency; metabolic labeling using glycans often occurs at multiple sites. In the work reported here, a simultaneous dual labeling system with high labeling efficiency and minimal tag size was developed. This was achieved by exploiting the different substrate specificities of the prenyltransferases, rat PFTase (rPFTase) and rat GGTase-I (rGGTase-I). By selecting CaaX sequences and isoprenoid analogues that are specific to distinct prenyltransferases, simultaneous selective labeling was accomplished. Using alkyne-containing and azide-containing isoprenoid analogues, protein tail-to-tail dimers were also created in a one-pot procedure,

Results and Discussion

1. Analysis of isoprenoid analogues as substrates for GGTase-I

In previous work, we and others have reported that isoprenoid analogues with various functional groups can serve as substrates for PFTase and be successfully incorporated into proteins.^{18–20,35,36} In contrast, the isoprenoid specificity of GGTase-I had not been explored in detail. Since GGTase-I is an essential enzyme in the prenylation process, the development of isoprenoid analogue substrates for GGTase-I is critical for probing and studying the protein substrates of GGTase-I in cells.²⁹ Therefore, we first studied the isoprenoid substrate specificity of rGGTase-I using a library of isoprenoid analogues (Figure 2). Based on crystallographic data, it is known that rGGTase-I has a larger isoprenoid substrate-binding pocket compared to that of PFTase.²⁴ Thus, we hypothesized that isoprenoid analogues with bulky moieties would be better recognized by rGGTase-I. Eight isoprenoid analogues that varied in the length of the carbon backbone and the size of additional functionality were screened as substrates for rGGTase-I using a green fluorescent protein modified with the C-terminal sequence, CVLL (GFP-CVLL, **9a**) as the protein substrate; CVLL is a naturally occurring rGGTase-I recognition sequence that has relatively high reaction efficiency compared to other rGGTase-I substrates.^{24,37} For the isoprenoid analogues, the syntheses of all except compound **2** have been previously described. The aminoxy-containing analogue (**2**) was prepared from an aldehyde precursor similar to **1** (Scheme S1) and was found to be a substrate for yeast PFTase (yPFTase) (Figure S1–S3). For the other isoprenoids, compounds **1**, **3**, **4** and **7** were previously known to be substrates for yPFTase^{18,35,38} and compounds **5** and **6** were known substrates for both yPFTase and rPFTase.³⁹ Compound **8** was a known substrate for GGTase-I.²⁹ LC-MS was used to determine the extent of prenylation of GFP-CVLL catalyzed by rGGTase-I with all these analogues and the reaction efficiency was calculated from the relative deconvoluted mass peak intensities of the prenylated and the unprenylated protein. Based on the mass spectrometry results (Table 1), we found that compound **3**, which contained a bulky aromatic moiety, as well as compounds **6** and **8**, which contained a longer isoprenoid backbone, were efficiently incorporated into GFP-CVLL (**9a**) by rGGTase-I. In contrast, analogues that were similar in length to FPP showed little or no product formation. These results were consistent with our hypothesis that the larger substrate-binding site of rGGTase-I would accommodate bulkier isoprenoid substrates.

To further study the binding of these analogues to rGGTase-I, we also performed computational docking experiments using analogues **3** and **8**. The structure of rGGTase-I bound with both GGPP and a geranylgeranylated peptide (KCVIL) product was used for that work. It was found that the isoprenoid backbone of analogue **8** adopted a similar “L” shape comparable to that of GGPP and that the fourth isoprenoid unit was oriented approximately 90° relative to the axis formed from the remaining isoprenoids, which was also consistent with the GGPP-bound structure (Figure 3A). For analogue **3**, due to the rigidity of the benzene ring, it adopted a different conformation than the corresponding isoprenoid units in the GGPP structure (Figure 3B). The root-mean-square deviation (RMSD) was calculated to quantify the differences between the conformations of GGPP and the docked analogues when docked into the rGGTase-I active site. The first two isoprenoid units of these

compounds were compared because they are closest to the site of reaction and because they have been found to undergo major conformational changes during the prenylation reaction.^{24,40–42} The RMSD between the first two isoprenoid groups (relative to bound GGPP) was calculated to be 1.09 Å for analogue **8** and 0.73 Å for analogue **3**. These small RMSD values suggest that the isoprenoid groups in these analogues can adopt similar conformations compared with GGPP and may account for their relatively high incorporation efficiency by rGGTase-I.

2. Identification of isoprenoid substrates that can be used for simultaneous dual protein labeling

Due to the different substrate specificities of rGGTase-I and rPFTase, we hypothesized that these two enzymes could be used to simultaneously label two different proteins (Figure 4). To investigate this, we sought to first identify a combination of a CaaX sequence and an isoprenoid analogue, which could be processed selectively by rGGTase-I, even in the presence of rPFTase. Similarly, it was envisioned that another combination of a CaaX sequence and an isoprenoid analogue could serve as a selective rPFTase substrate pair. Thus, when the two proteins with different CaaX motifs and the two isoprenoid analogues were combined in the presence of both enzymes, only two labeled protein products would form, with no cross reactions occurring.

GFP, whose fluorescence can be easily detected, was chosen as a model protein to characterize the dual labeling system. GFP appended with a C-terminal CVIA sequence (GFP-CVIA, **10a**), was used as the substrate for rPFTase since the CVIA motif is efficiently recognized by rPFTase.³⁹ GFP-CVLL (**9a**), described above, was used for rGGTase-I prenylation since it is also known to be a good substrate. Based on the screening results described above (Table 1), compounds **3** and **6** were first chosen as possible candidates for rGGTase-I substrates for the dual labeling system due to their high incorporation efficiency.

In order to determine whether these rGGTase-I isoprenoid substrates would fit the desired criteria of orthogonal reactivity, their inability to be incorporated by rPFTase needed to be ascertained. Therefore prenylation reactions containing analogues **3** and **6** were carried out with rPFTase and GFP-CVIA (**10a**). LC-MS was again employed to detect the presence of putative prenylated products and to quantify the extent of prenylation (Table 2). Analogue **6** was found to be incorporated into GFP-CVIA (**10a**) by rPFTase, which was consistent with previously reported results.³⁹ However, rPFTase unable to efficiently utilize analogue **3** in the prenylation reaction. Therefore, compound **3**, which was an efficient substrate for rGGTase-I but not for rPFTase was selected to used as the rGGTase-I complementary substrate in our dual labeling study.

To select an isoprenoid analogue for rPFTase prenylation reaction, compound **5**, which is a known rPFTase substrate,³⁹ was chosen because of its low rGGTase-I prenylation efficiency (Table 1) and orthogonal functionality relative to **3**. Reaction of analogue **5** with GFP-CVIA (**10a**) and rPFTase proved to be highly efficient (Figure S4), which supported this selection.

After choosing the respective isoprenoid substrates for rGGTase-I and rPFTase in the dual labeling system, it was also necessary to evaluate the cross reactivity of the enzymes to

prenylate the non-complementary CaaX sequence under the reaction conditions. To study this, four prenylation reactions were performed, including two rGGTase-I reactions with GFP-CVIA (**10a**) using **3** or **5** respectively, and two reactions for rPFTase with GFP-CVLL (**9a**) in the presence of either **3** or **5** (Table S2). The reactions with rGGTase-I showed no prenylated product with the noncognate substrate **10a**, confirming the high selectivity of rGGTase-I for its CaaX substrate. For the reactions containing rPFTase, it was found that although no prenylated product was produced with GFP-CVLL (**9a**) and **3**, minor amounts of **9a** modified with analogue **5** were detected via LC-MS. These results suggested that if GFP-CVIA (**10a**), GFP-CVLL (**9a**) and **5** were added together with rPFTase using the same reaction conditions as above, it would result in both proteins being modified with **5**, producing a mixture that was not desirable for dual labeling. Therefore, further investigation was needed to optimize the reaction conditions to achieve more specific labeling.

3. Optimizing reaction conditions to achieve specific labeling

In order to optimize the prenylation reaction conditions, we employed two dansylated peptide substrates, dansyl-GCVLL (Ds-GCVLL, **11a**) and dansyl-GCVIA (Ds-GCVIA, **12a**) (Figure 5A); these peptides and the resulting prenylated products can be readily monitored using reverse phase HPLC with fluorescence detection. The peptides and their prenylated products also have distinct retention times allowing the respective reaction efficiencies to be easily determined along with any cross-reaction that may occur.

Initial reactions using rPFTase with **11a**, **12a** and **5** showed two product peaks corresponding to **11c** and **12b** (Figure S5), in concordance with the LC-MS data described above. Previous CaaX substrate screening of rPFTase with peptide libraries have reported that the CVLL motif is a much poorer substrate compared to CVIA.³⁹ Therefore, it was likely that **11c** was formed here due to a high rPFTase concentration. When the concentration of rPFTase was reduced from 150 nM to 30 nM, only one product peak corresponding to **12b**, was observed (Figure 5B, ii). Thus, by reducing the concentration of rPFTase, we were able to selectively label only **12a** in the presence of the other CaaX substrate. For the reaction of rGGTase-I and **3** in the presence of both **11a** and **12a**, only one product, **11b**, was produced (Figure 5B, iii), indicating a high degree of selectivity for this reaction.

Finally, an all-in-one reaction was performed containing both prenyltransferases, both peptide substrates and both isoprenoid analogues. Under these optimized conditions, it was found that only the two desired products, **11b** and **12b**, were formed with no signs of cross prenylation (Figure 5B, iv). Thus, Ds-GCVIA (**12a**) was only prenylated with **5** by rPFTase and Ds-GCVLL (**11a**) was only labeled with **3** by rGGTase-I, demonstrating that the dual labeling system can be applied to peptide models successfully.

4. Simultaneous dual labeling on protein models

After performing proof-of-concept experiments with peptide substrates, the selective dual labeling strategy was applied to full-length proteins. GFP-CVLL (**9a**) and red fluorescent protein (TagRFP)⁴³ modified with a C-terminal CVIA motif (RFP-CVIA, **13a**) were chosen as convenient model proteins due to their different fluorescence excitation and emission

profiles. Using the optimized conditions obtained from the HPLC experiments described above, an all-in-one reaction with rPFTase, rGGTase-I, **3**, **5**, GFP-CVLL (**9a**) and RFP-CVIA (**13a**) was performed (Figure 6). It was found that only two prenylated products, ketone-labeled GFP-CVLL (**9b**) and alkyne-modified RFP-CVIA (**13b**) were produced. Using LC-MS, the labeling efficiency for rPFTase was estimated to be >99% and 52% for the rGGTase-I reaction. Thus, we were able to demonstrate that the dual labeling system can be applied to both large proteins and small peptides. The incorporated alkyne and ketone functionalities are not only bioorthogonal but they can also participate in different reactions: the copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction for the alkyne functional group and the oxime ligation for the ketone functional group. Therefore, subsequent ligation reactions can be performed in both parallel and sequential manners, showing significant potential for pulling down multiple proteins simultaneously and for creating patterned protein microarrays. Moreover, with the development of other orthogonal reaction pairs that can be performed simultaneously without cross-reactivity, such as the strain-promoted alkyne-azide cycloaddition (SPAAC) reaction and the tetrazine ligation,^{33,44–47} more isoprenoid analogues can be designed and potentially used in the dual labeling system, further expanding its application.

5. Protein dimerization using the CuAAC reaction

As noted above, LC-MS analysis (Table 1) showed that analogue **8** was efficiently utilized by rGGTase-I. Further investigation of this compound established that it was not recognized by rPFTase. Therefore, we expected that analogue **8** could be utilized, in place of **3**, in dual labeling experiments. By using analogues **5** and **8**, the two target proteins would be selectively modified with alkyne and azide functionality respectively, which could then be coupled together in a tail-to-tail manner via a one-pot procedure using the CuAAC reaction.

This strategy was first used to prepare a GFP-GFP homodimer (Figure 7). A single prenylation reaction was performed containing GFP-CVLL (**9a**), GFP-CVIA (**10a**), **5**, **8**, rGGTase-I and rPFTase. LC-MS results confirmed that only the azide-appended **9d** and alkyne-modified **10b** were produced. Although no product from cross prenylation was formed, the reaction efficiency for rPFTase-catalyzing reaction was only 36% (Figure S6). Since GGPP is known to inhibit the activity of rPFTase,⁴⁸ it is likely that compound **8** is also an inhibitor of rPFTase, given their similar structures. To overcome the inhibition effect, the amount of rPFTase used in the reaction was doubled. This led to complete conversion of GFP-CVIA (**10a**) to **10b** without the formation of the cross-prenylated product. The labeling efficiency for GFP-CVLL (**9a**) with analogue **8** by rGGTase-I was estimated to be 62%.

Once the specificity of the azide-alkyne dual labeling system was established, the CuAAC reaction was performed to produce GFP-GFP tail-to-tail homodimer. This was accomplished by the simple addition of the reagents (CuSO₄, TCEP and TBTA) to the reaction mixture following a desalting step to remove unreacted prenyl donor analogues **5** and **8**. SDS-PAGE and in-gel fluorescence imaging were employed to characterize the dimer (Figure 8). Importantly, to prevent the loss of GFP fluorescence due to complete denaturation, the protein samples were mixed with loading buffer and then applied directly onto an SDS-

PAGE gel without heating (Figure S7). The appearance of the dimer band, with higher molecular weight, was clearly observed in the CuAAC reaction mixture (Figure 8, Lane 4), indicating the successful formation of the GFP-GFP dimer. The reaction efficiency was estimated to be 50% from the gel image (Figure 8A, Lane 4). To further confirm that the desired cyclization reaction had occurred, the protein dimer was purified using size exclusion chromatography and characterized by LC-MS, which showed an observed mass of 56873 Da, closely matching the calculated mass of 56872 Da.

Beyond the GFP-GFP homodimer described above, we also wanted to demonstrate the generality of the dual labeling method by preparing a heterodimeric construct as well. Thus a chimeric protein incorporating red and green fluorescent proteins was targeted. For this, GFP-CVLL (**9a**), RFP-CVIA (**13a**), **5**, **8** and both prenyltransferases were incubated together. According to the LC-MS results, compound **5** was selectively incorporated onto RFP-CVIA (**13a**) by rPFTase with complete conversion and compound **8** was specifically appended to GFP-CVLL (**9a**) by rGGTase-I with 60% efficiency (Figure S8). No cross-reaction was found to occur. After prenylation and removal of unreacted prenyl donors, the CuAAC reaction was initiated by adding the reagents necessary for cycloaddition (CuSO₄, TCEP and TBTA). Protein aggregates were observed, which was probably due to the near neutral isoelectric point of the GFP-RFP dimer, but we were still able to analyze the reaction mixture using both SDS-PAGE and in-gel fluorescence imaging analysis. From the in-gel fluorescence imaging results (Figure 9), the fluorescence signal of the dimer band, which also manifested the expected increase in molecular weight, was detected when scanning for both GFP and RFP fluorescence, suggesting the successful dimerization of **9d** and **13b**. The molecular weight of the dimer was estimated to be 57.9 kDa based on SDS-PAGE analysis, which is in good agreement with the calculated mass of 57.5 kDa. The GFP-RFP dimer was also prepared using **9d** and **13b** that had each been generated separately and it was interesting to note that the CuAAC reaction efficiency of the simultaneously labeled **9d** and **13b**, estimated to be 23% based on band intensity (Figure S9B, Lane 6), was comparable to the 29% of the separately labeled proteins (Figure S9B, Lane 8). Since the yield of dimeric material was comparable whether dimerization was performed via simultaneous labeling (where incomplete prenylation occurred) or separate labeling (where the reactions were driven to completion), these results suggest that other factors such as dimer solubility likely play a role in influencing the yield in this particular case. Overall, with the utilization of the azide-containing and the alkyne-containing isoprenoid analogues, homo- and hetero-dimeric tail-to-tail protein constructs were successfully constructed in a one-pot procedure using the simultaneous dual labeling system.

Conclusion

In this work, we examined the isoprenoid substrate specificity of rGGTase-I using eight different isoprenoid analogues that incorporate various bioorthogonal functionality. In contrast to results obtained using PFTase, it was found that isoprenoid analogues with bulky moieties, such as a benzyl-ketone moiety, and a longer carbon backbone can be efficiently recognized by rGGTase-I. By taking advantage of the differences in both isoprenoid and protein substrates between rGGTase-I and rPFTase, we have successfully developed a simultaneous dual labeling system that can be used to selectively label two proteins in

tandem. After optimization of the reaction conditions, it was demonstrated that ketone functionality can be incorporated into GFP-CVLL specifically by rGGTase-I and an alkyne functional group can be attached to RFP-CVIA by rPFTase, with no cross reactivity between the enzymes and their substrates; subsequent work demonstrated that this system is not limited to only one isoprenoid substrate pair. Using azide- and alkyne-functionalized analogues, tail-to-tail homo- and hetero-dimeric proteins were produced in one-pot labeling reactions via this simple procedure. It should be noted that while the one-pot procedure developed here for dual labeling is typically less efficient than running the reactions separately due to incomplete conversion, the ability to perform these reactions simultaneously opens up the possibility of carrying them out in live cells. Thus, these *in vitro* biochemical experiments set the stage for future *in vivo* applications including *in vivo* heterodimerization and the generation of large multi-domain fusion proteins that may be otherwise difficult to express. Moreover, the tail-to-tail protein orientation generated via this strategy is not readily accessible via standard genetic methods. Finally, it should be noted that the ability to simultaneously label PFTase and GGTase-I substrates with orthogonal functionality should also be useful in prenylomic studies since it potentially allows levels of farnesylated and geranylgeranylated proteins to be studied concurrently. Overall, this dual labeling system greatly extends the utility of protein modification using prenyltransferases and opens up numerous avenues for future applications.

Materials and Methods

General

The synthesis for analogue **2** is provided in the supporting information. The remaining isoprenoid analogues employed here were synthesized as previously reported.^{18,20,29,35,36,38} Reagents were obtained from Sigma Aldrich unless otherwise noted. OD₆₀₀ of cells was taken with a DU 720 UV/Vis spectrophotometer (Beckman Coulter). Protein concentration was determined using a standard Bradford assay with a DTX 880 Multimode Detector plate reader (Beckman Coulter). The HPLC-based reaction condition optimization was performed using a Beckman model 125 solvent module coupled to a 305 fluorescence detector (Chrom Tech) and a Varian C₁₈ column (Microsorb-MW 5 μ m, 4.6 $\times$ 250 mm). LC-MS for the study of rGGTase-I isoprenoid substrate specificity was performed on a Synapt G2 mass spectrometer coupled to an Acquity ultra performance LC (Waters). LC-MS analysis for all other reactions was carried out using an Agilent 1100 series LC/MSD trap SL mass spectrometer. FPLC purification of GFP-GFP dimer was conducted with an AKTApurifier (GE Healthcare) and a HiPrep 26/60 Sephacryl S-100 column (GE Healthcare). Cells and proteins were mixed with 50% glycerol before storage at -80°C .

GFP-CVIA (10a) expression and purification

The vector (pJexpress414) expressing GFP-CVIA (**10a**) with a polyhistidine-tag was obtained from DNA 2.0 Inc. and was transformed into BL21(DE3) supercompetent cells (Novagen). The detailed sequence information is provided in the supporting information. To express the protein, BL21(DE3) cells were spread on LB-agar plates containing 100 $\mu\text{g/mL}$ ampicillin for overnight incubation at 37°C . A single colony was used to inoculate 50 mL of LB media containing 100 $\mu\text{g/mL}$ ampicillin and grown overnight at 37°C , shaking at 250

rpm. 10 mL of solution from the culture was then added to 1 L of LB media containing 100 µg/mL ampicillin, which was then incubated at 37 °C and shaking at 250 rpm. When the OD₆₀₀ of the media reached to 0.6, IPTG was added to a final concentration of 0.5 mM to induce protein expression at 25 °C overnight, shaken at 250 rpm. Cells were harvested by centrifugation at 5,400 g for 10 min and were then stored at –80 °C.

Nickel affinity chromatography was utilized to purify GFP-CVIA (**10a**). The cell pellet from 1 L of LB media was suspended in 50 mL lysis buffer (20 mM NaH₂PO₄, pH 8.0, 500 mM NaCl and 10 mM imidazole). Protease inhibitor cocktail for histidine-tagged proteins (1 mL, Sigma Aldrich) was also added to the buffer. The cells were lysed by sonication for 5 min (10 s on/10 s off) followed by clarification by centrifugation at 13,000 g for 30 min. The resulting clear supernatant was collected and loaded to a column with 25 mL of Ni-NTA resin (Qiagen), pre-equilibrated with wash buffer (20 mM NaH₂PO₄, pH 8.0, 500 mM NaCl and 20 mM imidazole) at a flow rate of 1 mL/min. The column was washed until the A₂₈₀ showed no further decrease. Protein was then eluted with elution buffer (20 mM NaH₂PO₄, pH 8.0, 500 mM NaCl and 250 mM imidazole) and green fractions were collected. All the green fractions were combined and concentrated to 2 mL by centrifugation using an Amicon Ultra-15 centrifugal filter (10 kDa cutoff, Millipore). Buffer exchange was performed by adding Tris-HCl buffer (50 mM, pH 7.3) to the concentrated solution and then concentrating to 2 mL again. The buffer exchange process was repeated two more times followed by storage at –80 °C in 50% glycerol prior to use. The concentration of GFP-CVIA (**10a**) was determined by its absorbance at 488 nm ($\epsilon=55,000 \text{ M}^{-1}\text{cm}^{-1}$).

Site-directed mutagenesis to produce GFP-CVLL (**9a**)

GFP-CVLL (**9a**) protein was produced by site-directed mutagenesis of DNA isolated from a clone producing GFP-CVIA (**10a**). The QuikChange II Site Directed Mutagenesis Kit protocol (Agilent, Catalog #200523) was followed and the primer sequences for PCR were determined using the QuikChange primer design tool. The sequence for the forward primer was GAC GAA CTG TAT AAG TGC GTG CTC TTA TAA AAG CTT CCC CCT AGC AT and the reverse primer was ATG CTA GGG GGA AGC TTT TAT AAG AGC ACG CAC TTA TAC AGT TCG TC. PCR amplification, DpnI DNA digestion and *E. coli* transformation were all carried out according to the QuikChange protocol. The plasmid expressing GFP-CVIA (**10a**) was extracted from BL21(DE3) cells and purified using a Wizard *Plus* SV Miniprep DNA Purification Kit (Promega, Catalog #A1460). The presence of the desired GFP-CVLL (**9a**) mutation was confirmed by DNA sequencing and the plasmid was then used to transform BL21(DE3) supercompetent cells (Novagen). The same procedure was employed to express and purify GFP-CVLL (**9a**) as was described above for GFP-CVIA (**10a**).

rPFTase expression and purification

rPFTase was expressed and purified following a previously reported procedure⁴⁹ and is similar to that described for GFP proteins. Streptomycin (50 µg/mL) was used as the selection antibiotic for cell growth. IPTG and ZnSO₄ were added, both with final concentration of 0.5 mM to induce protein expression. To purify the enzymes, lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 µM ZnCl₂, 5 mM MgCl₂, 20 mM imidazole)

and elution buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 μ M ZnCl₂, 5 mM MgCl₂, 250 mM imidazole) were used. 1 mM β -mercaptoethanol was added to these two buffers before use. Fractions containing rPFTase were collected and concentrated. Buffer exchange was performed with lysis buffer and the rPFTase was stored in 50% glycerol at -80°C .

rGGTase-I expression and purification

rGGTase-I was expressed in BL21(DE3) *E. coli* using a previously described pET23a rGGTase-I vector.⁵⁰ Bacterial cells transformed with the rGGTase-I expression vector were grown in autoinduction media (1% tryptone, 0.5% yeast extract, 25 mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 2 mM MgSO₄, 0.2x trace metals (0.01 mM FeCl₃, 0.004 mM CaCl₂, 0.002 mM MnCl₂, 0.002 mM ZnSO₄), 0.5% glycerol, 0.05% glucose, 0.2% α -D-Lactose) containing 100 μ g/mL ampicillin at 28°C for 24 hours.⁵¹

rGGTase-I was purified as previously reported, with dialysis steps replaced by desalting using a HiTrap desalting column (GE Healthcare).^{22,50} Expressed rGGTase-I from the bacterial lysate was purified by FPLC (AktaPrime), first using a HiTrap DEAE-Sepharose fast flow anion exchange column (GE Healthcare) over a linear gradient of 0–0.5 M NaCl. The fractions were tested for the presence of rGGTase-I by SDS-PAGE, with fractions containing rGGTase-I pooled and concentrated using Pall Macrosep advanced centrifugal devices (10 kDa molecular weight cut-off). The protein was run through a HiTrap desalting column (GE healthcare) using HTZ buffer (50 mM HEPES, pH 7.8, 1 mM TCEP, 10 μ M ZnCl₂) then through a HiTrap Q-sepharose HP anion exchange column (GE Healthcare) over a linear gradient of 0–0.5 M NaCl. Fractions containing rGGTase-I were collected and concentrated as before. Purified enzyme was then aliquoted, flash frozen in liquid nitrogen, and stored at -80°C . Enzyme concentration was measured by active-site titration using dansylated peptides, as described previously.^{22,37}

RFP-CVIA (13a) expression and purification

A C-terminal CVIA motif was appended to TagRFP through one round of PCR using the TagRFP-N vector (Evrogen) as a template, with the CVIA motif and stop codon encoded in the 3' primer upstream of a *Hind*III restriction site. The sequence for the forward primer containing an *Eco*RI restriction site and the 5' end of TagRFP was G CAG AAT TCG ATG GTG TCT AAG GGC GAA G, and the reverse primer containing the CVIA 3' extension and the *Hind*III restriction site was AAA CTG GGG CAC AAA CTT AAT GGT TGC GTA ATC GCT TGA AAG CTT TCG A. DNA primers were commercially synthesized (Integrative DNA Technologies, Coralville, IA). The PCR reaction (50 μ L) contained Standard OneTaq buffer (10 μ L), 10mM dNTPs (1 μ L), reverse primer (125 ng), forward primer (125 ng), template plasmid (10 ng), and OneTaq DNA polymerase (0.25 μ L, 5U/ μ L; New England BioLabs). PCR was performed on BioRad MyCycler under the following conditions: Initial denaturation (94°C , 1 min); thirty cycles of denaturation (94°C , 30s), annealing (56°C , 1min), and extension (68°C , 2min); final extension (68°C , 5min); and a final hold (10°C , ∞). After PCR, PCR products were purified using EZ-10 Spin Column PCR Purification Kit (BIO Basic Inc.) following the manufacturer's instructions.

A double digestion was then performed for ligation of EcoRI-TagRFP-CVIA-HindIII into CDF-Duet-1 plasmid and consisted of 10x NEB buffer 4 (2 μ L), 100x BSA (0.2 μ L), CDF-Duet-1 plasmid or PCR product (3 μ g), and restriction enzymes (NEB) EcoRI (1 μ L, 20 U/ μ L) and HindIII (1 μ L, 20U/ μ L). Digests were incubated for 2 hours at 37 °C. Digested DNA was then purified using 0.8% agarose gel (1x TAE buffer, 120 V). DNA was excised from the gel and purified using EZ-10 Spin Column DNA Gel Extraction Kit (BIO Basic Inc.) following the manufacturer's instructions.

Ligation reactions were carried out using the Quick Ligation Kit (NEB) following the manufacturer's protocol. An aliquot of the ligation reaction (5 μ L) was transformed into chemically competent DH5 α *E. coli* cells (Zymo research) before plating on LB-Strep plates and incubating overnight at 37 °C. Colonies were then inoculated into 5 mL cultures of LB media containing 100 μ g/mL streptomycin. Cultures were incubated and shaken at 225 rpm overnight at 37 °C. Plasmid DNA was then purified using EZ-10 Spin Column Plasmid DNA Minipreps Kit (BIO Basic Inc.) following the manufacturer's protocol. Successful ligation was confirmed through an analytical digest using EcoRI and HindIII restriction enzymes and the appearance of bands matching the size of the insert (750 bp) on 0.8% agarose gel (1x TAE buffer, 120V).

The CDF-Duet-1 vector containing the gene for RFP-CVIA (**13a**) with an N-terminal polyhistidine-tag was transformed into BL21(DE3) competent cells. The cells were spread on LB-agar plates containing 100 μ g/mL streptomycin for overnight incubation at 37 °C. A single colony was used to inoculate 5 mL of LB media containing 100 μ g/mL streptomycin and grown overnight at 37 °C, with shaking at 225 rpm. The 5 mL culture was then added to 1 L of LB media containing 100 μ g/mL streptomycin, and incubated at 37 °C, shaking at 225 rpm. When the OD₆₀₀ of the media reached to 0.7, IPTG was added to a final concentration of 0.5 mM and protein expression was induced at 28 °C overnight, shaking at 225 rpm. Cells were harvested by centrifugation at 6,000 g for 15 min and then stored at -80 °C.

RFP-CVIA (**13a**) was purified by nickel affinity chromatography. The cell pellet was suspended in 50 mL lysis buffer (20 mM NaH₂PO₄, pH 8.0, 500 mM NaCl and 10 mM imidazole). Benzonase (0.625U/50mL), lysozyme (0.25mg/mL), and PMSF (17 μ g/mL) were added to the cell suspension and incubated at 37 °C for 45 min. The cells were lysed on ice by sonication for 8 min (30 s on/30 s off) followed by clarification by centrifugation at 13,000 g for 30 min. The resulting clear supernatant was filtered through a 0.45 μ m filter and loaded onto a HisTrap HP 5 mL column (GE Healthcare), 3 columns in tandem, pre-equilibrated with wash buffer (20 mM NaH₂PO₄, pH 8.0, 500 mM NaCl and 20 mM imidazole) at a flow rate of 5 mL/min. The column was washed for 30 min. Protein was then eluted with elution buffer (20 mM NaH₂PO₄, pH 8.0, 500 mM NaCl and 250 mM imidazole) and red fractions were collected. All the red fractions (6 total, 2.5 mL each) were combined and concentrated to 2 mL by centrifugation using a Pall Macrosep Advance Centrifugal Device (10 kDa molecular weight cut-off). Buffer exchange was performed by adding Tris-HCl buffer (50 mM, pH 7.3) to the concentrated solution and then concentrating to 2 mL again. The buffer exchange process was repeated two more times followed by

storage at -80°C in 50% glycerol prior to use. The concentration of RFP-CVIA (**13a**) was determined by its absorbance at 555 nm ($\epsilon=100,000\text{ M}^{-1}\text{cm}^{-1}$).

Isoprenoid substrate analysis using rGGTase-I

A total of eight isoprenoid analogues (compounds **1** to **8**) were examined as substrates for rGGTase-I. Initial efforts using a continuous spectrofluorimetric assay to screen several isoprenoids as possible substrates for rGGTase-I using the dansyl-GCVLL peptide substrate **11a** proved to be difficult due to the lack of changes in dansyl fluorescence intensities for some of the analogues (Table S1). Next, the analogues were evaluated using a full-length protein substrate and LC-MS was employed to detect the prenylated product. The prenylation reaction was carried out for each analogue and for each reaction, a cocktail (250 μL) was prepared containing Tris-HCl (50 mM, pH 7.5), KCl (20 mM), MgCl_2 (10 mM), ZnCl_2 (10 μM), DTT (5 mM), and GFP-CVLL (**9a**, 2.5 μM). All these reagents were incubated on ice for 0.5 h. Next, isoprenoid analogue (5 μM , except 20 μM for **8**) and rGGTase-I (200 nM) were added to initiate each reaction which was then allowed to proceed in a 32°C water bath overnight followed by injection into the LC-MS instrument directly without any purification.

Prenylation reaction for rPFTase using analogue 5 as the isoprenoid substrate

A reaction cocktail (250 μL) was prepared containing Tris-HCl (50 mM, pH 7.5), KCl (20 mM), MgCl_2 (10 mM), ZnCl_2 (10 μM), DTT (5 mM), and GFP-CVIA (**10a**, 2.5 μM). All these reagents were incubated on ice for 0.5 h followed by the addition of analogue **5** (15 μM) and rPFTase (150 nM). The reaction was allowed to proceed in a 32°C water bath overnight followed by injection into the LC-MS instrument directly without any purification.

Docking of isoprenoid analogues to rGGTase-I

The docking experiments were performed using Maestro (Schrodinger, version 10.1). PDB file 1N4S was used as the rGGTase-I structure. Standard protein preparation was conducted using the default settings in the protein preparation wizard of the software. A receptor grid was generated. Glide docking was performed using the standard precision docking parameters and 10,000 ligand poses were run per docking for each analogue. Analogues **3** and **8** were docked into the substrate-binding site of rGGTase-I respectively and the conformation with the highest docking score was shown for each compound. RMSD was calculated using the RMSD calculation script from Schrodinger.

HPLC-based reaction condition optimization

Ds-CVLL (**11a**) and Ds-CVIA (**12a**) were synthesized as previously reported.⁵² A standard chromatogram for **11a** and **12a** was obtained with 1 mL of solution containing **11a** and **12a** (2.5 μM each), which were incubated with DTT (5 mM) for 1 h at RT before injection to insure complete thiol reduction. For rPFTase prenylation, a reaction cocktail (1 mL) was prepared containing Tris-HCl (50 mM, pH 7.5), KCl (20 mM), MgCl_2 (10 mM), ZnCl_2 (10 μM), DTT (5 mM) and was pre-incubated on ice for 0.5 h with **11a** and **12a** (2.5 μM each). Next, **5** (15 μM) and rPFTase (30–150 nM) were added to initiate the prenylation reaction.

The reaction mixture was incubated for 6 h in 32 °C water bath before HPLC injection for product analysis. The rGGTase-I prenylation reaction was carried out following the same procedure except that **3** (5 µM) and rGGTase-I (200 nM) were used instead. For the dual labeling experiment, **11a** and **12a** (2.5 µM each), **5** (15 µM), **3** (5 µM), rPFTase (30 nM) and rGGTase-I (200 nM) were added to the prenylation reaction. HPLC mobile phases were buffer A (0.1% aqueous TFA) and buffer B (CH₃CN with 0.1% TFA). A method with a flow rate of 1 mL/min and a gradient of 0–100% buffer B in 100 min was used for separation. Dansyl fluorescence was detected by excitation at 340 nm and emission at 550 nm.

Simultaneous dual protein labeling with **3** and **5**

A reaction cocktail (250 µL) was prepared with Tris·HCl (50 mM, pH 7.5), KCl (20 mM), MgCl₂ (10 mM), ZnCl₂ (10 µM), DTT (5 mM), GFP-CVLL (**9a**, 2.5 µM) and RFP-CVIA (**13a**, 2.5 µM). The mixture was incubated on ice for 0.5 h and the reaction was initiated by the addition of **3** (15 µM), **5** (5 µM), rGGTase-I (200 nM) and rPFTase (30 nM) and allowed to proceed overnight in a 32 °C water bath. Samples were removed and injected into the LC-MS instrument directly without any purification.

Simultaneous dual protein labeling with **3** and **8** and protein dimerization

The prenylation reaction cocktail (2 mL) was the same as the one described above except that **8** (20 µM) and rPFTase (60 nM) were used. Excess **5** and **8** were removed with a NAP-5 column (Amersham) using phosphate buffer (PB, 100 mM) as the eluent. The resulting solution was concentrated by ultrafiltration (Amicon Ultra-0.5 centrifugal filter, 10kDa cutoff, Millipore) to 50 µL and stored as a stock solution of labeled proteins (100 µM) at 4 °C. The CuAAC reaction (20 µL) was carried out with labeled proteins (50 µM), TBTA (100 µM from a 10 mM stock in 4:1 *t*-butanol:DMSO), TCEP (1 mM from a freshly prepared 50 mM stock) and CuSO₄ (1 mM). In the negative control experiment for the CuAAC reaction, an equal volume of the PB (100 mM) was added in lieu of CuSO₄. GFP-RFP protein dimers were also prepared using proteins that had been labeled individually via the same procedure except that each protein was first labeled and purified separately and then added to the CuAAC reaction mixture. The GFP-GFP dimer was purified by size exclusion chromatography using FPLC before LC-MS analysis. The column was pre-equilibrated with 2 column volumes of the buffer solution containing PB (50 mM, pH 7.0), NaCl (150 mM), DTT (5 mM) with a flow rate of 0.5 mL/min. Fractions for each peak were collected and analyzed by SDS-PAGE and LC-MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PFTase	protein farnesyltransferase
GGTase-I	geranylgeranyltransferase type I
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
yPFTase	yeast PFTase
rPFTase	rat PFTase
rGGTase-I	rat GGTase-I
GFP	green fluorescent protein
RFP	red fluorescent protein
RMSD	the root-mean-square deviation
Ds-GCVLL	dansyl-GCVLL
Ds-GCVIA	dansyl-GCVIA
CuAAC	copper (I)-catalyzed alkyne-azide cycloaddition
SPAAC	strain-promoted alkyne-azide cycloaddition
TBTA	tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TCEP	tris(2-carboxyethyl)phosphine
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
PB	phosphate buffer
DTT	dithiothreitol
IPTG	isopropyl β -D-1-thiogalactopyranoside

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Figure 1.
The protein prenylation reaction catalyzed by either PFTase or GGTase-I.

Figure 2.
Structures of isoprenoid analogues used for the analysis of rGGTase-I substrate specificity.

Figure 3.

Computational modeling of analogues **3** and **8** docked into the isoprenoid substrate-binding site of rGGTase-I. (A) Overlay structure of crystal bound GGPP and the docked analogue **8**. (B) Overlay structure of crystal bound GGPP and the docked analogue **3**. Note the similarity in the conformation of the first two isoprenoid units compared to bound GGPP. Color scheme and representations: the binding pocket of rGGTase-I is presented as a transparent surface. The Zn²⁺ atom is shown as an orange sphere. GGPP is shown in stick format (C and H in white; O in red; P in orange) in both panels. Analogue **8** is presented as green sticks in panel A and analogue **3** is given as pink sticks in panel B. The conformation (pose) with highest docking score is shown for each analogue. The pdb file 1N4S²⁴ was used to generate these images.

Figure 4. Scheme for simultaneous dual labeling using rPFTase and rGGTase-I. Protein A is only modified with analogue A by rGGTase-I and protein B is only labeled with analogue B by rPFTase.

Figure 5.

Prenylation reactions of rPFTase and rGGTase-I monitored by HPLC. (A) Scheme for the dual labeling reaction of Ds-GCVLL (**11a**) and Ds-GCVIA (**12a**) with isoprenoid analogues **3** and **5** in the presence of rGGTase-I and rPFTase. All the possible prenylated products are shown. (B) HPLC traces of prenylation reactions for rPFTase and rGGTase-I. i).

Unmodified **11a** and **12a**. ii). Prenylation reaction of **11a** and **12a** with **5** in the presence of rPFTase. iii). Prenylation reaction for **11a** and **12a** with **3** in the presence of rGGTase-I. iv). All-in-one prenylation reaction with **11a**, **12a**, **3** and **5** in the presence of both rPFTase and rGGTase-I. For each reaction, only the desired products were formed with no signs of cross prenylation.

Figure 6.

Scheme for the simultaneous labeling of GFP-CVLL (9a) and RFP-CVIA (13a) with the ketone-containing isoprenoid analogue 3 and the alkyne-containing analogue 5 by rPFTase and rGGTase-I. GFP-CVLL (9a) was only modified with the ketone functionality by rGGTase-I and RFP-CVIA (13a) was only labeled with the alkyne functional group by rPFTase. *The observed mass for 13a differs from the calculated mass by 16 units, which is likely due to the methionine oxidation.

Figure 7.

Scheme for simultaneous dual labeling of GFP-CVLL (**9a**) and GFP-CVIA (**10a**) with azide-containing isoprenoid analogue **8** and alkyne-containing analogue **5** by rGGTase-I and rPFTase, followed by the CuAAC reaction to produce GFP-GFP homodimer.

Figure 8.

SDS-PAGE and in-gel fluorescence imaging analysis of GFP-GFP dimer. (A) Coomassie blue stained gel of protein samples that were heated prior to loading. (B) Coomassie blue stained gel of protein samples that were loaded without heating. (C) In-gel fluorescence imaging of GFP fluorescence. Lane 1: unmodified GFP-CVIA (**10a**). Lane 2: unmodified GFP-CVLL (**9a**). Lane 3: simultaneously labeled **10b** and **9d**. Lane 4: the CuAAC reaction mixture of simultaneously labeled **10b** and **9d** with TBTA (100 μ M), TCEP (1 mM) and CuSO₄ (1 mM). The dimer band is highlighted in the red box. Lane 5: the CuAAC reaction control for the simultaneously labeled **10b** and **9d** with no CuSO₄ added. Densitometry analysis was performed using ImageQuant TL (version 7.0). Samples in panel C were not heated prior to gel loading. Note that the apparent size of GFP changes upon heat denaturation (compare panels A and B).

Figure 9.

In-gel fluorescence imaging analysis of the GFP-RFP dimer. (A) RFP fluorescence imaging. (B) GFP fluorescence imaging. The GFP-RFP dimer exhibited fluorescence in both GFP and RFP fluorescence channels. Lane 1: unmodified RFP-CVIA (**13a**). Lane 2: unmodified GFP-CVLL (**9a**). Lane 3: **13b**, produced from reaction of **13a**, **5** and rPFTase. Lane 4: **9d**, produced from reaction of **9a**, **8** and rGGTase-I. Lane 5: simultaneously labeled **13b** and **9d**. Lane 6: the CuAAC reaction mixture of simultaneously labeled **13b** and **9d** with TBTA (100 μ M), TCEP (1 mM) and CuSO₄ (1 mM). Lane 7: the CuAAC reaction control for the simultaneously labeled **13b** and **9d** with no CuSO₄ added. Lane 8: the CuAAC reaction mixture of separately labeled **13b** and **9d**. Lane 9: the CuAAC reaction control for the separately labeled **13b** and **9d** with no CuSO₄ added. The dimer band is highlighted in the red box.

Table 1

Prenylated products detected by LC-MS for the reactions of rGGTase-I with GFP-CVLL (**9a**) and eight isoprenoid analogues.

Substrate	Calculated Mass	Observed Mass	Efficiency
GFP-CVLL (9a)	28200	28200	-
FPP	28404	28403	33%
GGPP	28472	28472	100%
C15-Ald (1)	28418	N. D.	-
C10-Aoxy (2)	28424	N. D.	-
C10-BK (3)	28498	28498	84%
C10-BA (4)	28484	28481	54%
C10-Alk (5)	28390	N. D.	-
C15-Alk (6)	28458	28457	77%
C15-Azi (7)	28447	28444	50%
C20-Azi (8)	28513	28517	83%

Table 2

Prenylated products detected by LC-MS for the reactions of rPFTase with GFP-CVIA (**10a**) using isoprenoid analogues **3** and **6**.

Isoprenoid Substrate	Calculated Mass	Observed Mass	Efficiency
GFP-CVIA (10a)	20158	28155	-
C10-BK (3)	28456	N. D.	-
C15-Alk (6)	28416	28413	23%