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Application of *meta*- and *para*-phenylenediamine as enhanced oxime ligation catalysts for protein labeling, PEGylation, immobilization and release

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Abstract

Meta- and *para*-phenylenediamines have recently been shown to catalyze oxime and hydrazone ligation reactions at rates much faster than aniline, a commonly used catalyst. Here, it is demonstrated how these new catalysts can be used in a generally applicable procedure for fluorescent labeling, PEGylation, immobilization and release of aldehyde and ketone functionalized proteins. The chemical orthogonality of phenylenediamine-catalyzed oxime ligation versus copper catalyzed click reaction has also been harnessed for simultaneous dual labeling of bifunctional proteins containing both aldehyde and alkyne groups in high yield.

Keywords

Oxime ligation; Hydrazone ligation; *meta*-Phenylenediamine; *para*-Phenylenediamine; Dual protein labeling

Introduction

Conjugation of various synthetic groups to biomolecules is of great importance in the area of biology and biotechnology. Modification of proteins with imaging and targeting agents (Alley et al., 2010) and polymers (Veronese and Pasut, 2005) can result in the development of pharmaceuticals and highly specific drugs, imaging tools, and improvement of protein pharmacokinetics. During the past decade, several bioorthogonal chemical reactions have been developed such as the copper dependent or independent azide-alkyne cycloaddition, Staudinger ligation, and inverse electron demand Diels-Alder reaction (Patterson et al., 2014). Among various types of bioconjugation reactions, oxime and hydrazone ligations have recently attracted much attention since these imine-based reactions can proceed under mild conditions using easily accessible reagents. More importantly, there have been a number of methods developed for incorporation of aldehyde or ketone functionalities into proteins for subsequent oxime and hydrazone formation (Rabuka et al., 2012) (Rashidian et al., 2010). Oxime and hydrazone ligations are relatively slow under physiological conditions. In the early 1960s, Jencks and coworkers (Cordes and Jencks, 1962) demonstrated that aniline can catalyze the formation of oximes and hydrazones. More recently, Dawson and coworkers (Dirksen et al., 2006) applied aniline-catalyzed oxime ligation for bioconjugation, which has attracted much attention in the field. In several cases, aniline-catalyzed oxime ligation has been employed to site-specifically modify proteins.

However, even using aniline as a catalyst, these reactions, particularly oxime formation, still suffer from slow kinetics especially when a ketone is being used or when hydrazone-oxime exchange is conducted. Moreover, aniline has limited water solubility (~100 mM), and hence it cannot be used in higher concentrations to overcome the slow kinetics of the reaction.

To address this issue, our group and others have recently reported the discovery more efficient catalysts for oxime ligation (Crisalli and Kool, 2013). In our work, promising results were obtained using, *meta*-phenylenediamine (mPDA) and *para*-phenylenediamine (pPDA) which manifested greater activities relative to aniline (Rashidian et al., 2013b). Using phenylenediamines at higher concentration ranges (100–500 mM, past the solubility limit of aniline) results in an order-of-magnitude increase in the rate compared to aniline.

The diamines mPDA and pPDA each have unique advantages and disadvantages. mPDA is able to catalyze the reaction slightly faster than pPDA, however it forms a more stable Schiff base and hence can block oxime or hydrazone formation when used at high concentrations. In contrast, pPDA, is less efficient than mPDA but its Schiff base is much less stable, enabling it to be used in high concentration without blocking the reaction. Importantly, the relatively high rate of oxidation of pPDA in aerobic solutions (especially in the presence of copper salts used for click reactions), makes it less useful for labeling reactions where prolonged incubations are required. Therefore, although these two catalysts can be used interchangeably in some cases, each of their unique characteristics should be considered when employing them as catalysts for oxime and hydrazine formation.

In this Unit, we describe several detailed procedures for using pPDA and mPDA as improved catalysts for protein labeling, PEGylation, and reversible immobilization through oxime and hydrazone ligations (Figure 1). Basic Protocol 1 and 2 describe a procedure for forming and analyzing fluorophore labeling and PEGylation of aldehyde and ketone functionalized proteins. Basic Protocol 3 describes using the catalysts to enhance the immobilization rate of an aldehyde-containing protein on hydrazide-functionalized agarose beads. The immobilized protein is then released back into solution through mPDA or pPDA catalyzed hydrazone-oxime exchange along with simultaneous labeling and PEGylation. In Basic Protocol 4, we describe a one-pot efficient method for simultaneous dual labeling of an aldehyde- and alkyne-functionalized protein through mPDA catalyzed oxime ligation and copper catalyzed click reaction. This latter approach greatly simplifies the preparation of multifunctional protein conjugates. All the aldehyde-, ketone- or alkyne-functionalized proteins described in this protocol were developed in the Distefano laboratory for labeling using protein farnesyltransferase. However these bioconjugation procedures are applicable to any bioconjugation reaction involving aldehyde- or ketone-functionalized macromolecules.

Basic Protocol 1: Rapid Labeling and PEGylation of Aldehyde-Functionalized Proteins using Phenylenediamines as Catalysts

In a previous protocol paper (Mahmoodi et al., 2013) we described a detailed procedure for exploiting protein farnesyltransferase to incorporate aldehyde functionality into proteins.

Here we describe a procedure for rapid fluorophore incorporation and PEGylation of a given aldehyde-functionalized protein using m- or p-phenylenediamines (mPDA and pPDA). In this protocol we use aldehyde-labeled GFP as the model protein (detailed description of its preparation is reported in the earlier paper in (Mahmoodi et al., 2013)) to ligate with aminooxy-HiLyteFluor488 (Anaspec) and aminooxy-PEG (mol. wt. 5,000; NOF Corp.). Although, this protocol focuses on GFP as a model protein, this procedure is potentially applicable for ligation of various types of aldehyde-functionalized proteins with any aminooxy-functionalized molecule of interest (Figure 2).

Materials

The preparation of GFP-aldehyde was carried out in the Distefano laboratory (*diste001@umn.edu*) and methods for its preparation are fully described in a previous protocol paper (Mahmoodi et al., 2013).

m-Phenylenediamine and p-Phenylenediamine (Aldrich)

Aminooxy-HiLyteFluor488 (Anaspec)

Aminooxy-PEG (mol. wt. 5,000; NOF Corp., <http://nofamerica.net/>)

50 mM Tris•HCl, pH 7.5

0.3 M Phosphate Buffer, pH 7.0

DMSO or DMF (Aldrich)

NAP-5 or PD-10 column (Amersham)

LC-MS instrument (Waters Synapt G2 Quadrupole TOF mass spectrometer instrument)

MALDI-MS instrument (Bruker MALDI TOF)

Additional reagents and equipment for analyzing the efficiency of ligation: SDS PAGE (Gallagher, 2006) and MALDI-MS (Carr and Annan, 2001) (Zhang et al., 2010) and (Mahmoodi et al., 2013).

Coupling reaction between aldehyde-labeled protein and aminooxy compound

1. Prepare a 5 mM stock solution of aminooxy reagent (HiLyteFluor488 or aminooxy-PEG) in DMSO/water/DMF on any appropriate solvent.
2. Prepare a 0.5 M solution of either pPDA or mPDA in 0.3 M phosphate buffer at pH 7.0.
3. Prepare a 50 μ M stock solution of GFP-aldehyde in 50 mM Tris•HCl, pH 7.5.
4. Use the stock solutions prepared in steps 1 and 3 to generate a solution containing $\times$ μ M protein and $3\times$ μ M of aminooxy reagent in 0.1 M phosphate buffer, pH 7. Typically the protein concentration ($\times$) ranges from 5 – 300 μ M. A recommended starting point is between 50 and 100 μ M.
5. Initiate the ligation by adding the catalyst (mPDA or pPDA, prepared in step 2) to a final concentration of 25–100 mM, from a stock solution to the solution prepared in step 4.

Note: In the case of using mPDA as the catalyst, make sure the ratio of [mPDA]/[aminoxy] is less than 25, otherwise the ligation reaction may be blocked due to the competitive Schiff base formation between mPDA and the protein-aldehyde.

6. Mix the solution gently for 5 seconds.
7. Allow the reaction to proceed with agitation for 15 min to 4 h; depending on the concentration of reagents used, the reaction rate will vary.
8. Apply the resulting solution onto a NAP-5 column (or PD-10) preequilibrated with 50 mM Tris•HCl, pH 7.5, and collect the eluate.

This step removes the salts, the catalyst and the unreacted fluorophore or PEG reagent.

9. Confirm the formation of the ligated product by SDS-PAGE (Figure 2) (Gallagher, 2006).

Load 3 to 5 µg of protein into each lane. The PEGylated protein will appear as a lower mobility band. The fluorophore labeled protein can be observed by fluorescence scanning of the gel.

10. Confirm the formation of product by MALDI-MS or LC-MS analysis (Carr and Annan, 2001) (Zhang et al., 2010).

Basic Protocol 2: Labeling and PEGylation of Ketone-Functionalized Proteins using Phenylenediamines as Catalysts

Generally, aminoxy and hydrazine ligation reaction with ketones occurs at much slower rates relative to aldehydes. This is due to the lower electrophilicity and higher steric hindrance of the carbonyl groups in ketones versus aldehydes. Therefore, this necessitates using more efficient catalysts for accelerating ketone aminoxy or hydrazide ligation. In this section, we describe a detailed procedure using mPDA and pPDA for catalyzing ligation between a ketone-labeled protein and an aminoxy-fluorophore as well as an aminoxy-PEG reagent. Here, a ketone labeled GFP is used as a model protein and aminoxy-HiLyteFluor488 (Anaspec) and aminoxy-PEG (mol. wt. 5,000; NOF Corp) as aminoxy reagents.

Materials

The preparation of GFP-ketone was carried out in the Distefano laboratory (*diste001@umn.edu*) using methods similar to those employed for the GFP-aldehyde described above in Protocol 1 starting with GFP-CVIA whose preparation is fully described in a previous protocol paper (Mahmoodi et al., 2013). The synthesis procedure of the ketone analogue used in this example and subsequent attachment to eGFP-CVIA is described in the support protocol 1.

All reagents needed for Basic Protocol 1

Coupling reaction between ketone-labeled protein and aminooxy compound

1. Prepare a 5 mM stock solution of aminooxy reagent (HiLyteFluor488 or aminooxy-PEG) in DMSO/water/DMF or any appropriate solvent.
2. Prepare a 0.5 M solution of either pPDA or mPDA in 0.3 M phosphate buffer at pH 7.0.
3. Prepare a 50 μ M stock solution of GFP-ketone in 50 mM Tris•HCl, pH 7.5.
4. Use the stock solutions prepared in steps 1 and 3 [*Author: steps 1 and 3?] to generate a solution containing $\times$ μ M protein and $3\times$ μ M of aminooxy reagent in 0.1 M phosphate buffer, pH 7. The protein concentration ($\times$) usually ranges from 5 – 300 μ M. A concentration of 50 – 100 μ M is recommended as a starting point..
5. Initiate the ligation by adding the catalyst (either mPDA or pPDA) up to a final concentration of 25–100 mM, from a stock solution to the solution prepared in step 4.

Note: In case of using mPDA as the catalyst, make sure the ratio of [mPDA]/[aminooxy] is less than 25 otherwise the ligation reaction may be blocked due to the competitive Schiff base formation between mPDA and the protein-ketone.

6. Mix the solution gently for 5 seconds.
7. Allow the reaction to proceed for 5–10 h.

Note: In this case, the reaction time is significantly longer than with protein-aldehydes due to the lower reactivity of the protein-ketone.

8. Apply the resulting solution onto a NAP-5 (or PD-10) column preequilibrated with 50 mM Tris•HCl, pH 7.5, and collect the eluate.

This step removes the salts, the catalyst and the unreacted fluorophore or PEG reagent.

9. Confirm the formation of the ligated product by SDS-PAGE analysis (Gallagher, 2006).

Load 3 to 5 μ g of protein into each lane. The PEGylated protein appeared as a lower mobility band. The fluorophore labeled protein can be observed by fluorescence scanning of the gel.

10. Confirm the formation of product by MALDI-MS or LC-MS analysis (Carr and Annan, 2001) (Zhang et al., 2010).

Basic Protocol 3: Protein Immobilization and Release from Agarose Beads through Oxime Ligation Catalyzed by mPDA or pPDA

Many protein labeling applications are challenging due to low target abundance and high levels of contaminating protein impurities. One strategy for addressing this issue is to specifically immobilize the target protein on a solid support to remove the impurities and

then release it into a medium of interest. In a previous protocol paper (Mahmoodi et al., 2013) and a related manuscript (Rashidian et al., 2012), we reported a procedure for using protein prenylation to specifically incorporate an aldehyde label into a protein of interest in cell lysate, immobilize it on hydrazide-functionalized agarose beads and then release it in labeled form through hydrazone-oxime exchange. Although, the process was successful, using aniline as the catalyst, the exchange step (release of the protein) required a long reaction time to obtain significant conversion. Here, the more efficient catalysts mPDA and pPDA were used to significantly increase the rate of this release step.

In this protocol, aldehyde-labeled GFP, present in *E. coli* cellular extract, is immobilized on hydrazide-functionalized agarose beads. Using, mPDA or pPDA as the ligation catalyst, the immobilized protein is then released back into the solution and is simultaneously labeled by addition of an aminoxy reagent. Hydrazone ligation is faster than oxime ligation. Thus, in the immobilization step, using mPDA, pPDA or aniline, hydrazone formation is almost complete in less than 30 min, so the choice of which catalyst to use is less critical.

Materials

The preparation of GFP-aldehyde was carried out in the Distefano laboratory (*diste001@umn.edu*) and methods for its preparation are fully described in a previous protocol paper (Mahmoodi et al., 2013).

m-Phenylenediamine or p-Phenylenediamine (Aldrich)

Aminoxy-HiLyteFluor488 (Anaspec)

Aminoxy-PEG (mol. wt. 5,000; NOF Corp., <http://nofamerica.net/>)

Hydrazide-functionalized agarose beads (Thermo Scientific)

0.1 M Sodium phosphate buffer, pH 7.0

50 mM Tris•HCl, pH 7.5

1 M KCl in 50 mM Tris•HCl, pH 7.5

DMSO or DMF (Aldrich)

Centrifuge (Beckman-Coulter)

NAP-5 or PD-10 column (Amersham)

LC-MS instrument (Waters Synapt G2 Quadrupole TOF mass spectrometer instrument)

MALDI-MS instrument (Bruker MALDI TOF)

Additional reagents and equipment for analyzing the efficiency of ligation: SDS PAGE (e.g. Gallagher 2006) and MALDI-MS (e.g. Carr and Annan, 1977 and Mahmoodi et al., 2013).

Immobilize aldehyde-GFP on hydrazide functionalized beads

- 1 Shake the bottle of hydrazide-functionalized agarose beads to create a homogenous suspension, then quickly pipet the desired volume (50 – 250 μ L, 3

– 5 hydrazide equivalents) of the suspended beads into two microcentrifuge tubes.

The volume of the hydrazide beads necessary to use depends on the hydrazide resin loading. It is recommended that 3–5 eq of hydrazide beads be used. One tube is for the actual experiment and the other is for a control experiment.

2 Centrifuge the suspension for 30 sec at 1000×g and then discard the supernatant.

3 Add 300 µL of 0.1 M phosphate buffer, vortex the mixture for 5 sec, and centrifuge for 30 sec at 1000×g. Again, discard the supernatant.

4 Repeat steps 2 and 3 two more times.

5 Add a solution of GFP-aldehyde in cell lysate to the experimental tube containing the agarose beads, and add the same volume of 2 µM pure unmodified GFP (Mahmoodi et al., 2013) to the control reaction tube.

The reaction containing pure unmodified GFP serves as a negative control experiment.

6 Add mPDA, pPDA or aniline to a final concentration of 5, 20 or 50 mM respectively.

Note: Since hydrazones are less stable than oximes, Schiff base formation with the catalyst can be a serious problem and hence, care should be taken into the amount of catalyst being used. Excess amounts of catalyst can result in complete blocking of the immobilization reaction.

7 Let the reaction proceed for 30 min to 1 h with constant agitation so that the beads are suspended in the solution and do not settle to the bottom of the tube.

Shaking is necessary for completion of this reaction. Since reaction occurs on the surface of the resin, if shaking is not performed, the resin will quickly settle.

8 Centrifuge the mixture for 30 sec at 1000×g, and remove and discard the supernatant.

If using GFP-aldehyde, at this point the solution should be colorless and the beads should be green.

9 Wash the beads three times with 0.1 M phosphate buffer, pH 7.0, and then three times with 1 M KCl/50 mM Tris•HCl, pH 7.5, using the volumes and centrifugation conditions described in step 2. Store the immobilized protein at 4 °C in 50 mM Tris•HCl, pH 7.5 for the next steps.

Washing with KCl helps to remove nonspecifically bound proteins.

At this point, the agarose beads in the experimental tube should be green and beads in the control tube should be completely colorless. If

the control tube remains green, more washing is needed. If none of them appear green, it means immobilization was not successful and has to be repeated. In that case, the GFP-aldehyde protein (or other aldehyde-containing protein) should be analyzed via LC-MS to confirm the presence of the aldehyde.

The immobilized protein can be frozen and stored at -80°C for at least 6 months.

Simultaneous labeling and release of immobilized GFP

- 11 Prepare 1 mL of solution containing:
 - 0.1 M phosphate buffer, pH 7.0
 - 1–10 mM aminooxy reagent (PEG or HiLyteFluor 488)
 - 50–500 mM mPDA or pPDA

The total volume of the solution can be varied depending on the desired final concentration of the released protein.
- 10 Let the reaction proceed for 2–6 h with constant agitation so that the beads are suspended in the solution and do not settle to the bottom of the tube.
- 12 Centrifuge the mixture for 30 sec at $1000\times g$.
- 13 Collect the supernatant, which contains GFP-PEG (or GFP-HiLyteFluor488) conjugate.
- 14 Apply the resulting solution onto a NAP-5 (or PD-10) column preequilibrated with 50 mM Tris•HCl, pH 7.5, and collect the eluate.
- 15 Analyze the sample by SDS-PAGE (Gallagher, 2006). The PEGylated protein can be detected by its mass difference relative to the non-modified protein with the heavier PEGylated protein migrating more slowly. The fluorescently labeled protein can be detected by obtaining a fluorescence image of the gel. In this case, the fluorescently labeled protein will appear in the fluorescence image whereas the unmodified polypeptide will not.
- 16 Confirm the formation of conjugate product by MALDI-MS or LC-MS analysis (Carr and Annan, 2001) (Zhang et al., 2010).

Basic Protocol 4: Simultaneous Dual Protein Labeling using Oxime Ligation and Click Reaction

In this protocol we describe a detailed procedure for simultaneous dual protein labeling using both a click reaction and an oxime ligation. The model protein in this case is an aldehyde-alkyne-modified form of GFP (Rashidian et al., 2013a) which is labeled with an aminooxy-PEG and an azido-TAMRA reagent. This protocol is potentially applicable to any type of protein that has both aldehyde and alkyne functionality (Figure 3).

Materials

The method for preparing the Aldehyde-alkyne-GFP is described in our recent paper (Rashidian et al., 2013a). The synthesis procedure of the aldehyde alkyne-FPP analogue used in this example and subsequent attachment to eGFP-CVIA is described in the support protocol 1.

m-Phenylenediamine or p-Phenylenediamine (Aldrich)

Aminooxy-PEG (mol. wt. 5,000; NOF Corp., <http://nofamerica.net/>)

Azido-TAMRA (Lumiprobe, <http://www.lumiprobe.com/>)

CuSO₄ (Aldrich)

Tris (2-carboxyethyl)phosphine hydrochloride (TCEP, Aldrich)

Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, Aldrich)

1 M phosphate buffer, pH 7.0

500 mM Tris•HCl, pH 7.5

NAP-5 or PD-10 column (GE Healthcare)

LC-MS instrument (Waters Synapt G2 Quadrupole TOF mass spectrometer instrument)

MALDI-MS instrument (Bruker MALDI TOF)

Additional reagents and equipment for analyzing the efficiency of ligation: SDS-PAGE (e.g. Gallagher 2006) and MALDI-MS (e.g. Carr and Annan, 1977 and (Mahmoodi et al., 2013)

1. Prepare 2 mM stock solution of azido-TAMRA in DMSO (store solution at –20 °C).
2. Prepare 20 mM stock solution of aminooxy PEG in Water (store solution at –20 °C).
3. Prepare a solution containing:
 - 50 µM protein (1 eq.)
 - 150 µM azido-TAMRA (3 eq.)
 - 150 µM aminooxy-PEG (3 eq.)
 - 1 mM CuSO₄
 - 1 mM TCEP
 - 100 µM TBTA
 - 40 mM mPDA
 - 0.1 M phosphate buffer, pH 7.0

Cu (I) formed under click reaction condition oxidizes pPDA and rapidly quenches its catalytic activity. Therefore, pPDA cannot be used as the

oxime ligation catalyst in this dual labeling reaction. See critical parameters for more descriptions.

4. Let the reaction proceed for 2 h at room temperature with gentle shaking.

Both reactions will proceed to > 90 % completion in this time.

5. Apply the solution onto a NAP-5 (or PD-10) column preequilibrated with 50 mM Tris•HCl, pH 7.5, and collect the eluate.
6. Analyze the sample by SDS-PAGE. As depicted in Figure 3, the PEGylation of protein can be confirmed by observing a lower mobility band with higher mass relative to the unmodified protein; the fluorescence labeling can be detected from a fluorescence scan of the gel.
7. Confirm the formation of the conjugated product by MALDI-MS or LC-MS analysis.

SUPPORT PROTOCOL 1 : Synthesis of farnesyl ketone diphosphate (compound 1) and enzymatic incorporation into proteins

In a previous protocol paper (Mahmoodi et al., 2013), we described a detailed procedure for the synthesis and enzymatic incorporation of aldehyde-containing analogue of farnesyl diphosphate (FPP) into proteins, through use of protein farnesyl transferase (PFTase). Here we describe a procedure for the synthesis of acetylbenzoyl oxy-geranyl diphosphate or FPP-ketone (**1**, Figure 5), which is a ketone-functionalized analogue of FPP. This compound can then be incorporated into proteins that contain C-terminal CaaX box sequence using PFTase (Figure 6). The steady state kinetic parameters of FPP-ketone (**1**) are presented in Figure 5 and the synthetic scheme is depicted in Figure 7.

Materials

Geraniol (Aldrich)
Dichloromethane (CH₂Cl₂)
3,4-Dihydropyran (Aldrich)
Pyridinium *p*-toluenesulfonate (PPTS; Aldrich)
Diethyl ether (Aldrich)
Sodium bicarbonate (NaHCO₃)
Sodium sulfate (Na₂SO₄)
Silica gel 60
tert-Butyl hydroperoxide (Aldrich)
Selenous acid (H₂SeO₃; Aldrich)
Salicylic acid (Aldrich)
Toluene

Ethyl acetate (EtOAc)
Hexanes (Hex)
Dimethylsulfoxide (DMSO)
Triethylamine (TEA; Aldrich)
4-Acetylbenzoic acid (Aldrich)
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)
Dimethylaminopyridine (DMAP; Aldrich)
Isopropyl alcohol
Trichloroacetonitrile (CCl_3CN)
Acetonitrile (CH_3CN)
Bis(triethylammonium) hydrogen phosphate $(\text{Et}_3\text{NH})_2\text{HPO}_4$
Ammonium bicarbonate (NH_4HCO_3)
Heavy water (D_2O)
Deuterated chloroform (CDCl_3)
Sodium pyrophosphate ($\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$)
PFTase stock solution (see Mahmoodi et al., 2013)
250 mL, 100 mL, 50 mL round-bottom flasks
Magnetic stirrer and stir bar
Büchi Rotavapor model R-114 or equivalent rotary evaporator
10 × 2-in. (25.4 × 5.0-cm) chromatography column(s)
TLC plates (silica gel 60 F-254; Merck)
Separatory funnel
Oil bath
Büchner funnel
0.45- μm syringe filter
30 °C incubator
Amicon Centriprep centrifugal filter (MWCO 10,000; Millipore)
Centrifuge (Beckman-Coulter)
HPLC instrument: Beckman model 127/166 equipped with a UV detector and a Phenomenex C18 column (Luna, 10 μm , 10 × 250 mm) with a 5-cm guard column
Electrospray ionization mass spectrometer (ESI-MS; Bruker BioTOF II)
Lyophilizer

500-MHz ^1H NMR instrument (Oxford VI-500 MHz)

NAP-5 column (Amersham)

LC-MS instrument (Waters Synapt G2 Quadrupole TOF mass spectrometer instrument)

MALDI-MS instrument (Bruker MALDI TOF)

Additional reagents and equipment for analyzing the efficiency of prenylation (Support Protocol 3), SDS-PAGE (e.g., Gallagher, 2006), and MALDI-MS (e.g., Carr and Annan, 1997)

Synthesis of compound 3

- 1 Add 6.2 g (40 mmol) geraniol, 5.0 g (60.0 mmol) dihydropyran and 1.0 g PPTS (4.0 mmol) in a 250 mL round bottom flask equipped with a magnetic stir bar.
- 2 Add 60 mL of CH_2Cl_2 to the flask and mix until all the solids are dissolved.
- 3 Let the reaction mixture stir for 4 h at room temperature.
- 4 Evaporate the solvent under reduced pressure using a rotary evaporator.
- 5 Add 100 mL diethyl ether to dissolve the crude mixture.
- 6 In a separatory funnel, wash the resulting solution with 50 mL with brine.
- 7 Dry the organic layer over Na_2SO_4 , and remove the drying agent by filtration.
- 8 Evaporate the solvent under reduced pressure using a rotary evaporator.
- 9 Load 150 g silica gel 60 in a 10×2 -in. (25.4×5.0 cm) column equilibrated with 5:2 (v/v) toluene /EtOAc (mobile phase).
- 10 Dissolve the sample in small amount of mobile phase (~ 5 mL) and load it onto the column. Elute with 6 column volumes of the mobile phase. Analyze the eluate as it emerges from the column by TLC (use mobile phase as solvent, $R_f = 0.60$). Combine the fractions that contain the product.
- 11 Evaporate the solvents using a rotary evaporator to yield 9.1 g of compound 3 (98% yield) as colorless oil. ^1H NMR (300 MHz, CDCl_3) δ 1.52–1.87 (m, 6H), 1.63 (s, 3H), 1.71 (s, 6H), 2.05–2.12 (m, 4H), 3.51–3.56 (m, 1H), 3.89–3.93 (m, 1H), 4.06 (dd, $J = 12.0, 6.0$, 1H), 4.26 (dd, $J = 12.0, 6.0$, 1H), 4.66 (t, $J = 6.0$, 1H), 5.12 (dd, $J = 6.0, 1.0$, 1H), 5.39 (dd, $J = 6.0, 1.0$, 1H).

Synthesis of compound 4

- 12 To a 100 mL round bottom flask, add 5.8 g (20.0 mmol) of compound 3, 8.0 mL of *tert*-butyl hydroperoxide (72.0 mmol), 52 mg H_2SeO_3 (0.4 mmol), and 280 mg salicylic acid (2.0 mmol).
- 13 Dissolve the mixture in 20 mL CH_2Cl_2 .
- 14 Stir the mixture for 20 h at room temperature.
- 15 Evaporate the solvent using a rotary evaporator.

- 16** Add toluene to the remaining crude mixture followed by evaporation in a rotary evaporator. Repeat this step three more times to remove all remaining *tert*-butyl hydroperoxide.
- Toluene forms a low boiling point azeotrope with *tert*-butyl hydroperoxide and thus facilitates its evaporation.
- 17** Dissolve the remaining residue in 40 mL of diethyl ether.
- 18** In a separatory funnel, wash the resulting solution with 40 mL of 1.0 M NaHCO₃ to remove excess H₂SeO₃.
- 19** Wash the organic phase with 40 mL of brine.
- 20** Dry the organic layer over Na₂SO₄, and remove the drying agent by filtration.
- 21** Evaporate the solvents using a rotary evaporator.
- 22** Load 150 g silica gel 60 in a 10 × 2-in. (25.4 × 5.0 cm) column equilibrated with 5:2 (v/v) toluene /EtOAc (mobile phase).
- 23** Dissolve the sample in a minimal amount of mobile phase (3–5 mL) and load it onto the column. Elute with 6 column volumes of the mobile phase. Analyze the eluate as it emerges from the column by TLC (use mobile phase as solvent, R_f = 0.20). Combine the fractions that contain product.
- 24** Evaporate the solvents in rotary evaporator to yield 2.8 g of compound **4** (56% yield) as a colorless oil. ¹H NMR (500 MHz, CDCl₃) δ 1.47–1.83 (m, 6H), 1.63 (s, 3H), 1.65 (s, 3H), 2.05 (t, *J* = 7.5, 2H), 2.14 (t, *J* = 7.0, 2H), 3.46–3.51 (m, 1H), 3.83–3.88 (m, 1H), 3.94 (s, 2H), 3.99 (dd, *J* = 12.0, 7.5, 1H), 4.21 (dd, *J* = 12.0, 6.5, 1H), 4.60 (t, *J* = 3.0, 1H), 5.34 (ddd, *J* = 7.5, 7.5, 1.0, 2H).

Synthesis of compound 1

- 25** Flame dry a 100 mL round bottom flask.
- 26** Add 40 mL CH₂Cl₂ into the flask.
- 27** Add 2.8 g (11.0 mmol) of compound **3**, 2.5 g (15.4 mmol) of 4-acetylbenzoic acid and 1.2 g of DMAP (9.9 mmol) to the flask.
- 28** Cool the flask in ice bath and let it stir for 10 min.
- 29** Add 3.4 g (22.0 mmol) of EDC to the flask.
- 30** Stir the solution at 0 °C for 1 h.
- 31** Check for reaction completion by TLC (2:1 Hex:EtOAc).
- 32** Evaporate the solvent in a rotary evaporator.
- 33** Dilute the resulting mixture in 100 mL EtOAc.
- 34** Using a separatory funnel, wash the solution with 40 mL of 5% aqueous HCl.
- 35** Check the pH of aqueous layer using pH paper.

- 36 Continue washing the organic layer until the pH of the aqueous layer remains acidic. Two cycles of washing usually suffices.
- 37 Wash the organic solution two times, each time with 20 mL of sat. NaHCO_3 .
- 38 Wash the organic solution with 20 mL of brine.
- 39 Dry the organic phase over Na_2SO_4 and filter the solvent.
- 40 Evaporate the solvent using a rotary evaporator.
- 41 Add 3.0 g (7.5 mmol) of this intermediate protected alcohol in a 100 mL round bottom flask. Retain a small sample of the crude intermediate for TLC analysis.
- 42 Add 40 mL of *i*-ProOH.
- 43 Add 60 mg of PPTS to the flask.
- 44 Reflux the solution for 4 h.
- 45 Confirm the completion of the reaction by TLC (2:1 Hex:EtOAc) using crude starting material saved as noted in step 41.
- The deprotection of the alcohol will result in a lower mobility spot on the TLC plate.
- Continue the reaction until TLC shows >95% conversion.
- 46 Dilute the solution by adding 100 mL EtOAc.
- 47 In a separatory funnel, wash the organic phase with 20 mL NaHCO_3 .
- 48 Wash the organic phase with 20 mL of brine.
- 49 Dry the organic layer over Na_2SO_4 .
- 50 Evaporate the organic solvent in a rotary evaporator to give 2.1 g of compound **5** as a pale yellow oil (90% yield).
- 51 Dissolve 0.5 g (1.6 mmol, 1 eq) of **5** in 948 μL of CCl_3CN (9.5 mmol, 6 eq).
- 52 Add 1.2 g (4.0 mmol, 3 eq) of $(\text{Et}_3\text{NH})_2\text{HPO}_4$ to 7.6 mL CH_3CN in a separate flask, and place the flask in a 30 °C water bath to dissolve the salt.
- 53 Use an addition funnel to add the resulting solution (from step 52) dropwise over 3 h to the mixture obtained from step 51, while it is stirring at room temperature.
- Slow addition is critical for obtaining the highest possible yield.
- 54 After the additions is complete, stir the mixture for an additional 15 min at room temperature.
- 55 Remove the solvent under reduced pressure using a rotary evaporator.
- 56 Add 15 mL of 25 mM aqueous NH_4CO_3 solution to the residue, which results in the formation of a precipitate.

- 57** Filter the solution via vacuum filtration using a Buchner funnel and collect the filtrate.
- 58** Filter the resulting solution through a syringe filter and then purify by RP-HPLC using a semi-preparative column and the following conditions:
- detection at 214 nm
 - flow rate 5.0 mL/min
 - 5-mL injection loop
 - solvent A, 25 mM NH_4HCO_3 in water
 - solvent B, CH_3CN
 - Elution time program:
 - Gradient 0% to 35% solvent B in 30 min
 - 35% to 100% solvent B in 5 min.
- Compound **1** elutes in ~ 30 % solvent B. As soon as a peak appears in that range, collect fractions every 2 min in test tubes.
- 62** Analyze the collected fractions by ESI-MS in negative ion mode looking for a calculated mass of $\text{C}_{19}\text{H}_{25}\text{O}_{10}\text{P}_2\text{[M-H]}^-$ 475.0928.
- 59** Combine all the product-containing fractions and pour them into a lyophilization jar, freeze in liquid nitrogen, and lyophilize for 24 h to obtain approximately 0.1 g of compound **1** as a white powder. ^1H NMR (500 MHz, D_2O) δ 1.54–1.56 (m, 6H), 1.95 (t, $J = 8.0$, 3H), 2.07 (q, $J = 7.5$, 3H), 2.50 (t, 3H), 4.29 (t, $J = 7.0$, 2H), 4.54 (s, 2H), 5.28 (t, 1H), 5.42 (t, 1H), 7.80 – 7.86 (m, 4H).

Measure the concentration of ketone-FPP analogue (1)

A similar protocol is described in our previous protocol paper (Mahmoodi et al., 2013), for measuring the concentration of FPP analogue solutions.

- 63** Dissolve the powder resulted from step 63 in D_2O .
- This is the purified solution of the final product, but the concentration has to be measured.
- 64** Prepare a 10 mM solution of $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ in D_2O .
- This is used as an internal standard to measure the phosphorous concentration in the solution.
- 65** Combine equal volumes of the solutions prepared in step 64 and 65 (0.5 mL of each) in an NMR tube. Acquire ^{31}P NMR with a relaxation time of 45 sec for each pulse. Use the integration ratio between the phosphate peak (from $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$) and the diphosphate peak from the FPP analogue to calculate the concentration of compound **1**.

Note that the singlet peak appearing at -6.7 ppm corresponds to the phosphorous atom of $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, and the two doublets are related to the diphosphate substructure of the FPP-ketone analogue (**1**).

Incorporation of FPP-ketone into GFP-CVIA

Here we describe the procedure for using yeast PFTase to incorporate FPP-ketone analogue (**1**) into GFP-CVIA as a model protein. A detailed procedure for expression of GFP-CVIA as well as yeast PFTase is described in our previous protocol (Mahmoodi et al., 2013). The conditions for the enzymatic reaction reported here are the same as those used for the introduction of FPP-aldehyde analogues described in our previous protocol (Mahmoodi et al., 2013).

- 66** Prepare 10 mL of the following solution in a 15 mL centrifuge tube:

50 mM Tris•HCl, pH7.5

10 mM MgCl_2

30 mM KCl

10 μM ZnCl_2

5 mM DTT

2.4 μM GFP-CVIA

30 to 50 μM FPP-ketone

80 to 200 nM PFTase

PFTase should be added last, in order to avoid losing enzyme activity before initiating the reaction.

- 67** Incubate the solution for 4 h in a 30 °C incubator.

- 68** Concentrate the ketone-modified protein by loading the solution into a centrifugal filter (10,000 MW cutoff) and centrifuge at $5300 \times g$ to reduce the volume to about 500 μL .

- 69** Load the resulting solution onto a NAP-5 (or PD-10) column preequilibrated with 50 mM Tris•HCl, pH 7.5. Elute the column with 1 mL of 50 mM Tris•HCl, pH 7.5, and collect the eluate.

This step removes salts and excess FPP-ketone.

- 70** Analyze the efficiency of incorporation of FPP-ketone to the GFP-CVIA by LC-MS analysis. A mass increase of 298.2 Da relative to wild type protein is expected to be observed.

SUPPORT PROTOCOL 2

Here we describe a procedure for synthesis of (2E,6E)-8-(3-ethynyl-5-formylphenoxy)-3,7-dimethylocta-2,6-dien-1-yl diphosphate or alkyne-FPP analogue (**2**, Figure 5). This compound can then be incorporated into proteins that contain C-terminal CaaX box

sequence using PFTase (Figure 8). The steady state kinetic parameters of **2** are presented in Figure 5 and the synthetic scheme is depicted in Figure 9.

Materials

Resin-bound PPh₃ (Aldrich)
Tetrabromomethane (CBr₄, Aldrich)
3,5-Dihydroxy-benzaldehyde (Ellanova laboratories)
Dichloromethane (CH₂Cl₂)
Trifluoromethanesulfonic anhydride ((CF₃SO₂)₂O, Aldrich)
Bis(triphenylphosphine)palladium(II) dichloride (Pd(Ph₃)₂Cl₂, Aldrich)
Copper (I) iodide (CuI, Aldrich)
3,4-Dihydropyran (Aldrich)
Pyridinium *p*-toluenesulfonate (PPTS; Aldrich)
Diethyl ether (Aldrich)
Sodium bicarbonate (NaHCO₃)
Sodium sulfate (Na₂SO₄)
Ethynyltrimethylsilane (Aldrich)
Silica gel 60
Ethyl acetate (EtOAc)
Hexanes (Hex)
Dimethylsulfoxide (DMSO)
Triethylamine (TEA; Aldrich)
Dimethylaminopyridine (DMAP; Aldrich)
Isopropyl alcohol
Trichloroacetonitrile (CCl₃CN)
Acetonitrile (CH₃CN)
Bis(triethylammonium) hydrogen phosphate (Et₃NH)₂HPO₄
Ammonium bicarbonate (NH₄HCO₃)
Heavy water (D₂O)
Deuterated chloroform (CDCl₃)
Sodium pyrophosphate (Na₂H₂P₂O₇)
PFTase stock solution (see Mahmoodi et al., 2013)
250 mL, 100 mL, 50 mL round bottom flasks

Magnetic stirrer and stir bar

Büchi Rotavapor model R-114 or equivalent rotary evaporator

10× 2-in. (25.4 × 5.0-cm) chromatography column(s)

TLC plates (silica gel 60 F-254; Merck)

Separatory funnel

Oil bath

Büchner funnel

0.45-µm syringe filter

30 °C incubator

Amicon Centrifrep centrifugal filter (MWCO 10,000; Millipore)

Centrifuge (Beckman-Coulter)

HPLC instrument: Beckman model 127/166 equipped with a UV detector and a Phenomenex C18 column (Luna, 10 µm, 10 × 250 mm) with a 5-cm guard column

Electro spray ionization mass spectrometer (ESI-MS; Bruker BioTOF II)

Lyophilizer

500-MHz ¹H NMR instrument (Oxford VI-500 MHz)

NAP-5 column (Amersham)

LC-MS instrument (Waters Synapt G2 Quadrupole TOF mass spectrometer instrument)

MALDI-MS instrument (Bruker MALDI TOF)

Additional reagents and equipment for analyzing the efficiency of prenylation (Support Protocol 3), SDS-PAGE (e.g., Gallagher, 2006), and MALDI-MS (e.g., Carr and Annan, 1997)

Synthesis of compound 6

- 1 Add 0.10 g (0.39 mmol) of compound 4 (see support protocol 1) in a 25 mL round bottom flask equipped with a magnetic stir bar.
- 2 Add 0.36 g (1.58 mmol) resin-bound PPh₃.
- 3 Add 0.51 g (1.58 mmol) CBr₄.
- 4 Dissolve the mixture in 10 mL CH₂Cl₂.
- 5 Stir the solution for 2 h in room temperature.
- 6 Filter the mixture through a C18 Sep-Pak column to remove excess CBr₄ and resin-bound PPh₃.
- 7 Evaporate the solvents under reduced pressure using a rotary evaporator, to afford 99.0 g (~ 80 % yield) of compound 6 as the product.

Synthesis of compound 8

- 8 Add 2.00 g (14.5 mmol) of 3,5-dihydroxy-benzaldehyde into a round bottom flask equipped with a stir bar.
- 9 Add 4.4 g (43.4 mmol) TEA.
- 10 Dissolve the mixture in 20 mL of CH₂Cl₂.
- 11 Seal the flask with a septum and purge it with Ar or N₂.
- 12 Cool the flask in an ice bath.
- 13 In a separate flask, dissolve 8.9 g (31.5 mmol) of (CF₃SO₂)₂O in 10 mL of CH₂Cl₂.
- 14 Use a syringe to slowly add the solution prepared in step 13 to the reaction mixture prepared in step 12, while it is stirring and kept in an ice bath.
- 15 Let the reaction mixture stir in an ice bath for 2 h.
- 16 Allow the reaction to warm up to the room temperature, then add 50 mL H₂O to the flask to quench the reaction.
- 17 Extract the product from the mixture using 2×30 mL of CH₂Cl₂.
- 18 Combine the organic layers and wash them with 30 mL of 1.0 M aqueous HCl, 30 mL H₂O and 30 mL brine.
- 19 Dry the resulting solution over Na₂SO₄, and evaporate the solvent under reduced pressure to yield a brown crude residue.
- 20 Load 50 g of silica gel in a column and equilibrate it with Hex/EtOAc (4:1, v/v) (mobile phase).
- 21 Dissolve the sample in a minimal amount of mobile phase (3–5 mL) and load it onto the column. Elute with 4 column volumes of the mobile phase. Analyze the eluate as it emerges from the column by TLC (use mobile phase as solvent). Combine the fractions that contain product.
- 22 Evaporate the solvent in a rotary evaporator to yield 3.86 g of pale yellow powder (67 % yield). ¹H NMR (δ, CDCl₃): 10.02 (s, 1H), 7.84 (d, *J* = 2.0 Hz, 2H), 7.48 (t, *J* = 2.2 Hz, 1H). ¹³C NMR (δ, CDCl₃): 187.53, 158.93, 150.05, 121.98, 120.77, 117.34.

Synthesis of compound 9

- 23 Dissolve 3.6 g (8.95 mmol) of compound 8 in 40 mL of THF and 20 mL of TEA.
- 24 Add 63.5 mg (0.09 mmol) Pd(Ph₃)₂Cl₂ to the reaction mixture.
- 25 Add 34.7 mg (0.18 mmol) CuI to the reaction mixture.
- 26 Purge the reaction flask with Ar or N₂ for 5 min.
- 27 Cool the reaction flask in ice bath at 0 °C.

- 28 Prepare a solution of 1.27 mL ethynyltrimethylsilane (8.95 mmol) in 20 mL THF, and use a syringe to slowly add it to the reaction mixture over 10 min.
- 29 Let the reaction stir for 2 h at room temperature.
- 30 Check the completion of the reaction by TLC (Hex:EtOAc, 30:1, v/v).
- 31 Remove the solvents under reduced pressure and diluted with 30 mL sat. NH_4Cl .
- 32 Extract the aqueous layer two times, each time with 50 mL EtOAc.
- 33 Combine organic layers and wash them with 50 mL H_2O , 50 mL of brine and then dry over Na_2SO_4 .
- 34 Remove the solvent under reduced pressure.
- 35 Load 50 g silica gel in a column and equilibrate it with Hex/EtOAc (30:1, v/v) (mobile phase).
- 36 Dissolve the sample in a minimal amount of mobile phase (~ 3 mL) and load it onto the column. Elute with 4 column volumes of the mobile phase. Analyze the eluate as it emerges from the column by TLC (use mobile phase as solvent). Combine the fractions that contain product.
- 37 Evaporate the solvent in a rotary evaporator to yield 1.72 g of pale yellow powder (55.5 % yield). ^1H NMR (δ , CDCl_3): 9.97 (s, 1H), 7.95 (d, J = 1.0 Hz, 1H), 7.70 (dd, J = 1.0, 1.0 Hz, 1H), 7.57 (dd, J = 1.0, 1.5 Hz, 1H), 0.254 (s, 9H). ^{13}C NMR (δ , CDCl_3): 189.19, 149.60, 138.10, 133.29, 129.71, 120.93, 100.94, 99.54, 79.80, 68.19, -0.383.

Synthesis of Compound 10

- 38 Dissolve 1.7 g (4.85 mmol) of compound **9** in 15 mL MeOH.
- 39 Cool the resulting solution in an ice bath to 0 °C.
- 40 Prepare a solution of 1.0 g LiOH in 15 mL MeOH, and add it to the solution prepared above.
- 41 Let the reaction stir overnight at room temperature.
- 42 Evaporate the solvent under reduced pressure.
- 43 Dilute the residue with 45 mL Et_2O .
- 44 Wash the organic layer with 10 mL H_2O , and 10 mL brine.
- 45 Dry the resulting solution over Na_2SO_4 .
- 46 Evaporate the solvent under reduced pressure.
- 47 Load 25 g silica gel in a column and equilibrate it with Hex/ Et_2O (2:1, v/v) (mobile phase).
- 48 Dissolve the sample in a minimal amount of mobile phase (3–5 mL) and load it onto the column. Elute with 4 column volumes of the mobile phase. Analyze the

eluate as it emerges from the column by TLC (use mobile phase as solvent). Combine the fractions that contain product.

- 49** Evaporate the solvent in a rotary evaporator to yield 0.55 g of pale yellow powder (77 % yield). ¹H NMR (δ, CDCl₃): 9.90 (s, 1H), 7.54 (dd, *J* = 1.5, 1.5 Hz, 1H), 7.34 (dd, *J* = 1.0, 1.0 Hz, 1H), 7.21 (dd, *J* = 1.0, 1.0 Hz, 1H), 6.09 (s, 1H), 3.13 (s, 1H); ¹³C NMR (δ, CDCl₃): 191.48, 156.35, 137.80, 127.06, 124.96, 115.34, 113.59, 81.80, 78.76.

Synthesis of compound 11

- 50** Flame dry a 50 mL flask.
- 51** Dissolve 0.36 g (1.14 mmol) of bromide **6** and 0.29 g (2.00 mmol) of **10** in 15 mL anhydrous DMF.
- 52** Add 0.50 g (3.62 mmol) of K₂CO₃.
- 53** Stir the reaction at 100 °C for 3 h.
- 54** Check for the completion of the reaction by TLC (Hex:EtOAc, 2:1, v/v).
- 55** Evaporate the solvent under reduced pressure.
- 56** Load 10 g silica gel into a column and equilibrate it with Hex/Et₂O (5:1, v/v) (mobile phase).
- 57** Dissolve the sample in a minimal amount of mobile phase (3–5 mL) and load it onto the column. Elute with 5 column volumes of the mobile phase using (Hex:Et₂O) from 1:0 (v/v) going to 5:1 (v/v). Analyze the eluate as it emerges from the column by TLC. Combine the fractions that contain product.
- 58** Evaporate the solvent in a rotary evaporator to yield 0.27 g (0.70 mmol) of pale yellow powder (77 % yield). ¹H NMR (500 MHz, CDCl₃) δ 9.91 (s, 1H), 7.54 (dd, *J* = 1.0 Hz, 1H), 7.37 (dd, *J* = 1.0 Hz, 1H), 7.26 (dd, *J* = 1.0 Hz, 1H), 5.53 (t, *J* = 7.0 Hz, 1H), 5.35 (t, *J* = 6.5 Hz, 1H), 4.61 (m, 1H), 4.42 (s, 2H), 4.24 (dd, *J* = 9.5, 6.5 Hz, 2H), 4.00 (m, 1H), 3.95–3.83 (m, 1H), 3.50 (m, 1H), 3.13 (s, 1H) 2.20 (m, 2H), 2.08 (t, *J* = 7.5 Hz, 2H), 1.71 (s, 3H), 1.67 (s, 3H), 1.66–1.46 (m, 5H). ¹³C NMR (125 MHz, CDCl₃) δ 191.13, 159.17, 139.85, 139.44, 137.63, 130.26, 129.16, 127.41, 126.77, 124.70, 120.98, 114.32, 97.81, 82.01, 78.46, 63.55, 62.24, 38.87, 30.64, 25.91, 25.42, 19.55, 16.35, 13.78.

Synthesis of Compound 12

- 59** Dissolve 0.25 g (0.65 mmol) of **11** in 15 mL *i*-PrOH in a round bottom flask.
- 60** Add 10 mg PPTS to the flask.
- 61** Reflux the reaction for 4 h.
- 62** Check for the completion of the reaction by TLC (Hex:EtOAc, 2:1, v/v).
- 63** Add 5 mL of saturated NaHCO₃ to the flask.
- 64** Add 50 mL of EtOAc to the flask.

- 65** Separate the organic layer using a separatory funnel.
- 66** Dry the organic layer with 10 mL brine and then over Na₂SO₄.
- 67** Remove the Na₂SO₄ by filtration and evaporate the solvent under reduced pressure to yield 49 mg of compound **12** (25 % yield) as pale yellow oil. The purity of the product that is obtained in this step is sufficient for the next step without chromatographic

purification. ¹H NMR (500 MHz, CDCl₃) δ 9.90 (s, 1H), 7.54 (dd, *J* = 1.0 Hz, 1H), 7.37 (dd, *J* = 1.0 Hz, 1H), 7.26 (dd, *J* = 1.0 Hz, 1H), 5.52 (t, *J* = 7.0 Hz, 1H), 5.39 (t, *J* = 6.5 Hz, 1H), 4.42 (s, 2H), 4.13 (d, *J* = 7.0, 2H), 3.12 (s, 1H) 2.20 (m, 2H), 2.06 (t, *J* = 7.0 Hz, 2H), 1.70 (s, 3H), 1.66 (s, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 191.48, 156.35, 139.64, 141.82, 137.80, 127.06, 124.96, 124.48, 115.34, 113.57, 81.80, 80.22, 78.76, 68.22, 65.99, 27.07, 22.82, 15.12, 14.68.

Synthesis of compound 2

The following steps are similar to related ones described in support protocol 1.

- 68** Dissolve 25 mg (0.08 mmol, 1 eq) of **12** in 50.5 μL of CCl₃CN (0.50 mmol, 6 eq).
- 69** Add 75.6 mg (0.25 mmol, 3 eq) of (Et₃NH)₂HPO₄ to 3.0 mL CH₃CN in a separate flask, and place the flask in a 30 °C water bath to dissolve the salt.
- 70** Use an addition funnel to add the resulting solution (from step 55) dropwise over 3 h to the mixture obtained from step 54, while it is stirring at room temperature.
- Slow addition is critical for obtaining the highest possible yield.
- 71** After the addition is complete, stir the mixture for an additional 50 min at room temperature.
- 72** Remove the solvent under reduced pressure using a rotary evaporator.
- 73** Add 5 mL of 25 mM aqueous NH₄CO₃ solution to the residue, which results in the formation of a precipitate.
- 74** Filter the solution via vacuum filtration using a Buchner funnel and collect the filtrate.
- 75** Filter the resulting solution through syringe filter and then purify by RP-HPLC using a semi-preparative column under the following conditions:
- detection at 214 nm
- flow rate 5.0 ml/min
- 5-ml injection loop
- solvent A, 25 mM NH₄HCO₃ in H₂O
- solvent B, CH₃CN

Elution time program:

Gradient 0% to 35% solvent B in 30 min

35% to 100% solvent B in 5 min.

Compound **2** elutes from ~ 20–25 % solvent B. As soon as a peak appears in that range, collect fractions every 2 min in test tubes.

- 71** Analyze the collected fractions by ESI-MS in negative ion mode looking for a calculated mass of $C_{19}H_{25}O_{10}P_2^-$ [M-H]⁻ 475.0928.
- 76** Combine all the product-containing fractions and pour them into a lyophilization jar, freeze in liquid nitrogen, and lyophilize for 24 h to obtain about 3.84 mg (10 % yield) of compound **2** as a white powder. ¹H NMR (500 MHz, CDCl₃) δ 9.71 (s, 1H), 7.55 (dd, *J* = 1.0 Hz, 1H), 7.37 (dd, *J* = 1.0 Hz, 1H), 7.30 (dd, *J* = 1.0 Hz, 1H), 5.46 (t, *J* = 7.0 Hz, 1H), 5.27 (t, *J* = 7.0 Hz, 1H), 4.43 (s, 2H), 4.28 (d, *J* = 6.5, 2H), 3.44 (s, 1H) 2.08 (m, 2H), 1.95 (t, *J* = 7.5 Hz, 2H), 1.55 (s, 3H), 1.53 (s, 3H). ³¹P NMR: (121 MHz, D₂O) δ -6.47 (d, *J* = 17.2, 1P), -3.51 (d, *J* = 16.5, 1P). HR-ESI-MS calcd for $C_{15}H_{26}O_8P_2$ [M-H]⁻ 395.1025, found 395.0907.

Measure the concentration of 3-ethynyl-5-formylphenoxy geranyl diphosphate (**2**)

A similar protocol has been described in our previous protocol paper (Mahmoodi et al., 2013), for measuring the concentration of FPP analogue solutions.

- 77** Dissolve the powder resulting from step 76 in D₂O.
- This is the purified solution of the final product, but the concentration has to be measured.
- 78** Prepare a 10 mM solution of Na₂H₂P₂O₇ in D₂O.
- This is used as an internal standard to measure the phosphorous concentration in the solution.
- 79** Combine equal volume of the solutions prepared in step 77 and 78 (0.5 mL of each) in an NMR tube. Acquire ³¹P NMR with a relaxation time of 45 sec for each pulse. Use the integration ratio between singlet peak and one of the doublets to calculate the concentration of compound **2**.
- Note that the singlet peak appearing at -6.7 ppm corresponds to the phosphorous atom of Na₂H₂P₂O₇, and the two doublets are derived from the 3-ethynyl-5-formylphenoxy geranyl diphosphate (**2**).

Incorporation of compound **2** into GFP-CVIA

- 80** Prepare 10 mL of the following solution in a 15 mL centrifuge tube:
- 50 mM Tris•HCl, pH7.5
- 10 mM MgCl₂
- 30 mM KCl

10 μ M ZnCl₂

5 mM DTT

2.4 μ M GFP-CVIA

30 – 60 μ M Compound **2**

80 to 200 nM PFTase

PFTase should be added last, in order to avoid losing enzyme activity before initiating the reaction.

- 81** Incubate the solution for 4 h in a 30 °C incubator.
- 82** Concentrate the ketone modified protein by loading the solution into a centrifugal filter (10,000 MWCO) and centrifuge at 5300 $\times$ g to reduce the volume to about 500 μ L.
- 83** Load the resulting solution onto a NAP-5 (or PD-10) column preequilibrated with 50 mM Tris•HCl, pH 7.5. Elute the column with 1 mL of 50 mM Tris•HCl, pH 7.5, and collect the eluate.
- This step removes salts and excess FPP analogue.
- 84** Analyze the efficiency of incorporation of FPP-ketone into the GFP-CVIA by LC-MS analysis. A mass increase of 280.4 Da relative to wild type protein is expected to be observed.

Commentary

Background information

Bioorthogonal reactions have wide ranging applications in the area of cellular biology, biotechnology, pharmaceuticals and tissue engineering. These chemistries can be used to label specific proteins to help understand cellular pathways (Chen and Ting, 2005), construct protein arrays for bioassays (Byeon et al., 2010), and develop antibody drug conjugates for cancer therapy (Krop et al., 2010).

Several useful bioorthogonal chemistries have been employed in the field of bioconjugation. Important examples are the inverse electron demand Diels-Alder reaction (Blackman et al., 2008), copper-catalyzed and copper-independent azide-alkyne cycloaddition (Bertozzi, 2011) and imine formation (Dirksen et al., 2006). Imine-based ligations are particularly useful because of the ease of synthesis of the constituents, commercial availability, minimal modification to the biomolecules, mild reaction conditions and more importantly their ease of incorporation into proteins and biomolecules. In the case of the inverse electron demand Diels-Alder reaction and the copper-free click reaction, both are highly efficient and do not need an added catalyst. However, both often require reagents that manifest limited solubility in water, thereby complicating their use. In contrast, oxime and hydrazone formation do not have these limitations.

Currently there are several methodologies available for the enzymatic incorporation of aldehydes and ketones into proteins, including the use of formyl-glycine-generating enzyme

described by Bertozzi and coworkers (Rabuka et al., 2012), incorporation of the non-natural amino acid, p-acetyl phenylalanine, reported by Schultz and coworkers (Wang and Schultz, 2005) and the use of aldehyde-containing isoprenoids developed by the Distefano group (Rashidian et al., 2013a, Rashidian et al., 2010). These methods make hydrazone and aminooxy ligation more readily accessible for a variety of applications.

The unique properties of oxime and hydrazone ligation encouraged our group and others (Crisalli and Kool, 2013) to work on discovering more efficient catalysts for this reaction and further broaden its application. In screening the catalytic activity of a series of phenylenediamines and aminophenols, we discovered that meta and para phenylenediamines have higher catalytic efficiency compared to aniline. These catalysts also possess significantly higher water solubility (~2 M) relative to aniline (~100 mM), allowing them to be used at higher concentrations resulting in faster reaction rates. Our data showed that while pPDA only slightly increases the reaction rate (compared with aniline, both at 100 mM), mPDA catalyzes the reaction twice as fast as aniline. However, if used at high concentrations, mPDA forms a relatively stable Schiff base with the aldehyde, leading to incomplete oxime formation. Therefore, we suggested that the mPDA/aminooxy ratio be kept below 250 to avoid this blocking effect. Apparently, this number is case-sensitive, since Wendeler et al. recently reported that they observed no product formation when performing oxime ligation using mPDA under conditions similar to those described above (Wendeler et al., 2014). Importantly, as suggested by them and from our data, for general oxime ligation reaction, pPDA can be used at high concentrations without any blocking effects, which can result in an improved reaction rate (Bindman and van der Donk, 2013). However, pPDA can be readily oxidized in aerobic solution particularly in the presence of copper ions (Geller et al., 1959), which makes it unusable for reactions that require prolonged reaction times or for dual labeling of aldehyde- and alkyne-functionalized proteins. Recently, Lee et al (Lee et al., 2014) showed that mPDA can significantly increase the rate of ketone hydrazine ligation in comparison with other catalysts. Moreover, mPDA-catalyzed oxime ligation can be performed at lower pH values resulting in much faster reaction rates, while pPDA may not be useful under such conditions due to its higher pK_a (pK_a = 4.9 for mPDA versus pK_a = 6.2 for pPDA). In conclusion, both mPDA and pPDA have specific advantages and disadvantages that need to be considered when choosing the catalyst for the oxime ligation reaction. However, if used appropriately, these catalysts can result in efficient and much faster conjugations relative to the traditional aniline-catalyzed reaction. In the following section, more details are provided to guide the selection of an appropriate catalyst for specific reaction conditions.

Critical Parameters and Troubleshooting

Reagent preparation and storage—Preparation of phenylenediamine stock solutions in phosphate buffer: mPDA is soluble up to 2 M. pPDA is less soluble and thus it is recommended that it be prepared as a 1 M stock solution. Sonication and heat up to 30°–40 °C is helpful for the dissolution of both catalysts. Phenylenediamines are prone to oxidation and lose their activity over time if exposed to air and light at room temperature. Therefore, to maximize catalysis, stock solutions should be stored at –20 °C and covered with aluminum foil to protect them from ambient light. The oxidation of pPDA occurs at much

higher rate compared with mPDA. A stock solution of pPDA darkens visibly even after only 2 h incubation at room temperature. It is recommended that a fresh pPDA solution be prepared for each use. All the proteins and protein conjugate solutions should be stored at -80°C for long-term storage. Avoid multiple freezing and thawing cycles for protein solutions since they may become denatured and/or inactive.

Catalyst selection—Since kinetic studies have shown similar catalytic efficiency for mPDA and pPDA, either of these two compounds can be used to catalyze oxime ligation. However, each has limitations that should be considered when using them under specific reaction conditions.

The main limitation of using mPDA is the formation of a relatively stable Schiff base with the aldehyde component within the reaction. Using large excesses of mPDA favors Schiff base formation over subsequent oxime ligation, leading to incomplete reaction. To prevent this blocking effect, the ratio of [mPDA]/[aminooxy Reagent] should not exceed 25. In contrast, pPDA has no such limitation and can be used in higher concentrations and results in faster reactions.

However, pPDA is oxidized in the presence of ambient oxygen at a relatively rapid rate and thus loses its catalytic activity. Exposure of pPDA solution to air results in a black solution in as little as two hours at room temperature. Therefore, in catalyzing aminooxy-ketone ligations, which may take more than 8 hours for completion, the pPDA concentration will decrease significantly over the course of the reaction. While this problem can be solved by performing the reaction under an inert atmosphere, in practice such operations are inconvenient. The rapid oxidation of pPDA is particularly troublesome for simultaneous protein labeling using a combination of click reaction and oxime ligation. Under conditions required for Cu-catalyzed click reaction, significant oxidation of pPDA occurs. For example, in the experiment shown in Figure 4, equal concentrations of the two catalysts (mPDA or pPDA) were utilized for ligating aminooxy-TAMRA to both ketone- and aldehyde-functionalized GFP in the presence of reagents needed for click chemistry. In experiment A, no copper was added to the reactions, thus both catalysts remained active, resulting in efficient formation of labeled GFP in all four reactions. pPDA catalytic efficiency was slightly higher as evidenced by the darker bands in the fluorescence channel image. However, addition of copper to the reactions significantly attenuated the catalytic effect of pPDA while having little effect on the mPDA-containing reaction. Based on the fluorescence image of the gel shown in Figure 4, the extent of labeling catalyzed by mPDA is significantly higher for both the ketone and aldehyde labeling reactions, indicating the catalytic activity of pPDA is substantially reduced due to oxidation by copper ions under those conditions.

Performing the experiment—After performing the protein immobilization step, it is important that the beads are washed with a 1 M KCl solution to insure that all nonspecifically bound proteins are removed.

Troubleshooting—If the coupling reaction is going to be performed on a new protein it is always helpful to perform a parallel reaction with a known protein using same conditions to

be used as a standard for testing the coupling conditions. Failure of the coupling reaction may be due to several reasons. The stock solution of the catalyst might be old and oxidized over time. Since phenylenediamines are relatively inexpensive, it is suggested to always prepare a fresh catalyst solution. If the mPDA is used as the catalyst, make sure to follow the critical ratio of catalyst/aminooxy reagent to avoid any blocking effect. This necessitates having relatively accurate concentration of all the reagents. Another source of problems is aldehyde-modified proteins which tend to oxidize to carboxylic acids over time. This can be easily verified by obtaining LC-MS of the protein, since the oxidation would increase the mass of the protein by 16 Da.

To troubleshoot the capture and release of non-fluorescent proteins, a parallel control reaction with a fluorescent protein (such as GFP-Aldehyde) can be performed. By keeping the reaction conditions identical in the two reactions, it is possible to follow the completion of each step by monitoring the immobilization and subsequent release of fluorescence of the control reaction.

Anticipated Results

Basic Protocols 1 & 2 generate fluorescently labeled and PEGylated proteins through oxime ligation using either pPDA or mPDA as the catalyst. In Basic Protocol 1, the reaction occurs between an aldehyde-functionalized protein and an aminooxy-reagent, which is expected to be complete in approximately 30 min to 4 h depending on the concentration of reagents used. However, in Basic Protocol 2, the reaction occurs between a ketone-functionalized protein and an aminooxy reagent, which requires 5–10 h of incubation. Labeled proteins can be verified by SDS-PAGE, LC-MS and MALDI-MS. The labeling methods described here are robust and reproducible, and can be exploited using various types of aldehyde- or ketone-bearing proteins. In Basic Protocol 3, an aldehyde-functionalized protein in a complex matrix (in this case, soluble cellular extract) is captured with hydrazide beads, released back into a solution of interest and simultaneously labeled or PEGylated by different aminooxy reagents. Using mPDA or pPDA as catalysts can significantly increase the rates of these reactions, especially the release step, which is the bottleneck for the whole process. Using this procedure, any aldehyde-labeled protein can be enriched, purified and finally released back into a solution of interest along with simultaneously being fluorescently labeled or PEGylated.

In Basic Protocol 4, an aldehyde- and alkyne-functionalized protein is simultaneously labeled with a fluorophore and a PEG chain in a one-pot reaction. The procedure is highly robust, proceeds to complete conversion in less than 2 h and can be used to incorporate multiple labels into proteins. Our laboratory has successfully employed this procedure for simultaneous labeling and PEGylation of CNTF protein, a protein of therapeutic interest (Rashidian et al., 2013a). Similar to previous protocols, products can be verified by SDS-PAGE or mass spectrometric techniques.

Time Considerations

Having all the small molecule reagents and the functionalized protein available, each of the protein modification procedures together with subsequent analysis can be accomplished in a

one or two day period. Aldehyde-functionalized proteins can be modified within a day whereas ketone-functionalized proteins require two days due to the lower reactivity of the ketone. The procedures described here use proteins that have been functionalized using PFTase in conjunction with aldehyde- or ketone-modified substrates. Such proteins can be prepared starting from purified unmodified protein and the desired substrate in a single day. The complete procedure for that is detailed in our previous protocol paper (Mahmoodi et al., 2013). Other methods for preparing aldehyde- or ketone-modified proteins take comparable amounts of time. The aldehyde- and ketone-containing FPP analogues used in the preparation of the above modified proteins typically can be prepared in 1–2 weeks. Importantly, solutions of these compounds, once prepared and standardized, are stable for at least a year if stored at -80°C . The quantities of these compounds prepared using the procedures outlined in this protocol are sufficient for a large number of protein labeling reactions. Hence, typically, it is only necessary to perform the synthetic chemistry described here one time.

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Figure 1.

Schematic representation of (A) oxime ligation reaction between an aldehyde- or ketone-functionalized protein and aminoxy reagent, catalyzed by *para*-phenylenediamine (pPDA). (B) Simultaneous dual labeling of an aldehyde alkyne-functionalized protein through copper-catalyzed click reaction and *meta*-phenylenediamine (mPDA)-catalyzed oxime ligation. In this protocol, the red rectangle symbolizes a HiLyteFluor 488 fluorophore or a PEG group while the pink circle symbolizes a TAMRA fluorophore.

Figure 2. (A) Coupling reaction between GFP-aldehyde and HiLyteFluor488 catalyzed by either mPDA or pPDA. (B) SDS PAGE analysis of the reaction: Lane 1 is pure GFP-aldehyde and lane 2 is fluorescently labeled protein. The left panel is visualized by Coomassie blue staining and the right panel is visualized by in-gel fluorescence imaging.

Figure 3.

(A) Schematic representation of oxime ligation and click reaction between aldehyde, alkyne-functionalized GFP with TAMRA-azide and aminoxy-PEG (3 kDa). (B) SDS-PAGE analysis of the reaction. Lower panel is visualized by Coomassie blue staining, while the bands in the upper panel are visualized by in-gel fluorescence imaging. Lane 1: Reaction mixture containing the bifunctional protein, TAMRA-azide and aminoxy PEG, Lane 2: Reaction mixture containing bifunctional protein and TAMRA-azide, Lane 3: Reaction mixture containing bifunctional protein and aminoxy PEG and Lane 4: Solution containing only bifunctional protein.

Compound	$k_{\text{cat}}(\text{s}^{-1})$	$K_{\text{M}}(\text{mM})$	$(k_{\text{cat}}/K_{\text{M}})_{\text{rel}}$
FPP	0.52	1.71	1
1	0.0028 ± 0.0002	2.57 ± 0.75	0.0036
2	0.012 ± 0.001	2.53 ± 0.34	0.016

Figure 4.

GFP-aldehyde or GFP-ketone (20 μM) was reacted with aminooxy-TAMRA (100 μM) using 25 mM of each catalyst in PB (100 mM, pH 7) containing TCEP (1 mM), TBTA (100 μM). The reaction was carried out in a final volume of 50 μL and was performed at room temperature for 15 h. In both panel A) and B) Lane 1: Marker (25 kDa), Lane 2: GFP, Lane 3, 4: GFP-ketone and Lane 5, 6: GFP-aldehyde. This data clearly indicates that pPDA activity is decreased in the presence of copper while mPDA is not.

Figure 5.
Steady state kinetic parameters of FPP analogues which are substrate for PFTase.

Figure 6.
Schematic representation of prenylation of a protein containing a C-terminal Caax box,
using FPP-ketone analogue which yields a ketone functionalized protein.

Figure 7.
Synthesis of farnesyl ketone diphosphate (**1**) from geraniol.

Figure 8.
Schematic representation of prenylation of a protein containing a C-terminal Caax box, using aldehyde alkyne-FPP analogue (2) which yields a ketone functionalized protein.

Figure 9. Synthesis of (2E,6E)-8-(3-ethynyl-5-formylphenoxy)-3,7-dimethylocta-2,6-dien-1-yl diphosphate or aldehyde alkyne-FPP analogue (2).