Synthetic Isoprenoid Analogues for the Study of Prenylated Proteins:

Fluorescent Imaging and Proteomic Applications

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ABSTRACT

Protein prenylation is a posttranslational modification catalyzed by prenyltransferases involving the attachment of farnesyl or geranylgeranyl groups to residues near the C-termini of proteins. This irreversible covalent modification is important for membrane localization and proper signal transduction. Here, the use of isoprenoid analogues for studying prenylated proteins is reviewed. First, experiments with analogues containing small fluorophores that are alternative substrates for prenyltransferases are described. Those analogues have been useful for quantifying binding affinity and for the production of fluorescently labeled proteins. Next, the use of analogues that incorporate biotin, bioorthogonal groups or antigenic moieties is described. Such probes have been particularly useful for identifying proteins that are naturally prenylated within mammalian cells. Overall, the use of isoprenoid analogues has contributed significantly to the understanding of protein prenylation.

KEYWORDS: bioorthogonal labeling, farnesylation, fluorescent isoprenoids, geranylgeranylation, prenylomics, protein prenylation, proteomics

Introduction to protein prenylation

After a new polypeptide is synthesized, the resulting protein is frequently altered in a process called post-translational modification. Examples of this include cleavage by a protease, phosphorylation by a kinase or lipidation by a prenyltransferase. The diverse roles of post-translational modification include modulating enzymatic activity, altering protein-protein interactions or changing cellular localization. Protein modification with isoprenoids has been the focus of numerous studies since its discovery in the early 1990's because of its connection to cancer.¹ Members of the Ras superfamily proteins are normally prenylated and mutated forms of Ras, especially K-Ras, are involved in as many as 30% of all human cancers.² Members of the protein prenyltransferase class of enzymes include protein farnesyltransferase (PFTase) and geranylgeranyltransferase I (GGTase I). These prenyltransferase enzymes are heterodimers containing a common α -subunit but different β -subunits that are only about 25% sequence identical.³ Most contacts between the substrates and the enzyme involve the β -subunit (Figure 1, Upper Panel).

Figure 1. Structure of PFTase. Upper Panel: Global structure of PFTase bound to the peptide CVIM and an isoprenoid analogue. Lower Panel: Expanded view of the isoprenoid analogue bound in the active site. Color scheme: α -subunit (magenta), β -subunit (white), CVIM peptide (yellow) and isoprenoid (C: green; N: blue; O: red; P: orange). For the expanded view, both subunits are shown in gray with C-12 of the isoprenoid highlighted in cyan. The peptide substrate has been removed to highlight the isoprenoid binding pocket. These images were created using the pdb file 1D8D.⁴

PFTase and GGTase-I catalyze the transfer of farnesyl (C15) and geranylgeranyl (C20) groups respectively, from the corresponding allylic diphosphates. In the resulting alkylated protein, the isoprenoid group is linked to a cysteine residue within the C-terminal amino acid sequence referred to as a Ca_1a_2X motif where C is a

cysteine, a_1 and a_2 are usually aliphatic amino acids and X is the major determinant for modification by either PFTase or GGTase I (Figure 2).⁵⁻⁷ It has been reported that farnesylation by PFTase occurs when X is alanine, serine, methionine or glutamine, while geranylgeranylation by GGTase I occurs when X is leucine or phenylalanine. Additional proteins are di-geranylgeranylated at their C-termini when they contain sequences including CC and CXC; these latter sequences are prenylated by protein geranylgeranyltransferase type II (GGTase-II). GGTase-II, also called RabGGTase, differs both structurally and functionally from the canonical PFTase and GGTase-I enzymes because it recognizes more extensive elements from its cognate protein substrates, Rab proteins.⁸ Once prenylated, the resulting proteins typically move to the endoplasmic reticulum where they may be further processed by the proteases Rce1p or Ste24p that remove the a_1a_2X tripeptide and methylated by a SAM-dependent methyltransferase (Icmt) to produce proteins with a C-terminal methyl ester (Figure 2).⁹

Figure 2. C-terminal prenylation and subsequent proteolysis and methylation. The prenyl group can be a farnesyl group (C_{15}) as shown here or a geranylgeranyl group (C_{20}). In the latter case, prenylation is catalyzed by GGTase-I or -II in lieu of PFTase.

Protein prenylation is not only common in mammals^{10,11} but is also a ubiquitous post-translational modification in all eukaryotes. For example, prenylated Ras is a more potent activator of *S. cerevisiae* adenylyl cyclase than is the non-prenylated form.¹² It has also been found that prenylation of signal transduction proteins is essential for the viability of *C. albicans* which is an opportunistic fungal pathogen.¹³ Other groups have identified prenylated proteins and confirmed their significance in *P. falciparum* which is the causative agent for malarial disease.¹⁴ A vast array of prenylation inhibitors have been developed to combat numerous illnesses caused by cancers, protozoan pathogens, and fungal infections.¹⁵⁻²⁰ Prenylation has also been found to be critical in plants.^{21,22} Recently, interest in prenylation has expanded to include biotechnology applications since prenyltransferases can be used to enzymatically incorporate non-natural functional groups into protein substrates.²³⁻²⁵

The resulting modified polypeptides can be further transformed via bio-orthogonal reactions to produce a variety of useful species including PEGylated proteins,²⁶ protein multimers²⁷ and protein-DNA conjugates.^{28,29} The focus of this review is on the use of isoprenoid analogues for the study of prenylated proteins. A wide variety of synthetic isoprenoids has been prepared to probe the specificity and mechanism of prenyltransferases,^{30,31} serve as inhibitors,³² and study prenylation in cells;³³ those topics are not covered here since a number of excellent reviews describing that work have already been published. Instead, the use of analogues containing fluorescent groups, labels for pull-down experiments and functional groups for bio-orthogonal reactions is the emphasis here since considerable progress has been made in this area in the last 10 years.³⁴

Fluorescent analogues

Since the native isoprenoid substrates of prenylation (FPP and GGPP) do not have particularly useful spectroscopic properties, this makes the monitoring of localization of intracellular proteins and their biochemical characterization difficult. Fluorescent derivatives, prepared via chemical synthesis (Figure 3) have been used to label proteins *in vitro* or in live cells and have thus been particularly useful for studying the interaction and subcellular localization of prenylated proteins.

Figure 3. Fluorescent analogues of FPP and GGPP used for the study of protein prenylation.

One approach to produce fluorescent FPP and GGPP analogues is to extend double bond conjugation; thus FPP analogue ($\Delta\Delta$ FPP, **1a**, and GGPP analogue ($\Delta\Delta$ GGPP, **1b**, were synthesized and studied.^{35,36} The alcohol form of **1b** ($\Delta\Delta$ GGOH) showed blue fluorescence in methanol, with $\lambda_{\text{ex}} = 310$ nm and $\lambda_{\text{em}} = 410$ nm but the fluorescence intensity at 410 nm was largely quenched in aqueous solution.

Side-chain prenylation of N-acetylated Cys methyl ester with **1b** showed $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 460$ nm in Tris buffer demonstrating the environmental sensitivity of the conjugated fluorophore. Compound **1b** is a substrate for yeast GGTase-I with a K_M value of $0.33 \mu\text{M}$ (compared with $3.45 \mu\text{M}$ for GGPP) but the prenyl-transfer efficiency (V_{max} in uncorrected fluorescence units) was lower than that of GGPP. The dissociation constant (K_D) of AcCys($\Delta\Delta$ GG)Me to RhoGDI, which forms a complex with geranylgeranylated Rho protein, is $15.1 \mu\text{M}$ ($2.45 \mu\text{M}$ for AcCys(GG)Me) indicating that the more rigid $\Delta\Delta$ GG analogue manifests lower affinity for the protein target. The alcohol of **1a** ($\Delta\Delta$ FOH) displayed blue fluorescence in methanol, with $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 465$ nm but again the fluorescence is quenched in aqueous solution. Compound **1a** is not a substrate for yeast PFTase but instead, is a potent competitive inhibitor (Table 1).

Beyond the above two unsaturated analogues, all of the subsequent fluorescent analogues shown in Figure 3 have been prepared by appending existing fluorophores onto the end of the isoprenoid chain placing them in the vicinity of C-12 of FPP (Figure 1, Lower Panel, C-12 colored in cyan). The first two examples of such analogues are the *N*-methylantranilate-labeled FPP analogue (**2a**) and GGPP analogue (**2b**).^{37,38} Both compounds are characterized by λ_{ex} at 340 nm and λ_{em} at 435 nm and both compounds are substrates for GGTase-II with **2b** binding better than **2a**

(compare K_D values of 330 nM and 49 nM for **2a** and **2b**, respectively) presumably because **2b** is structurally more similar to GGPP.

The first advantage of appending a fluorophore onto the isoprenoid chain is that it facilitates the determination of binding affinity and kinetics. Previous attempts to determine those properties depended on changes in the intrinsic tryptophan fluorescence in the active site but in many cases the intensity changes were small. If an extra fluorophore is attached to the isoprenoid chain and binding of the fluorescent derivatives to the enzymes results in significant fluorescence changes, binding affinity can be measured via fluorescence titration. Subsequent affinities of non-fluorescent substrates can be determined by competitive titrations. Thus, the binding constants of **2a** and **2b** for GGTase-II were determined in this manner. The transient kinetics of **2a**, **2b**, FPP and GGPP interaction with GGTase-II were determined similarly but the signal originated from fluorescence resonance energy transfer between a protein-derived tryptophan and the N-methylantranilate moiety present in the isoprenoid.

Although compound **2a** and **2b** have applicability in enzymatic assays, the ester linkage in the structure may be cleaved by nonspecific esterases in cellular experiments. A more stable anthranilate derivative with an amine linkage (**3**) should overcome this potential problem. Incubation of cells with compound **3** led to the

accumulation of non-prenylated Ras, which can be prevented by coincubation with FPP, indicating that compound **3** is likely a competitive inhibitor with respect to FPP.³⁹ The alcohol version of compound **3** is toxic to RPMI-8402 human leukemia cells with IC₅₀ of ~10 nM. The fluorescent analogue **4** and its alcohol version were also visualized within cells by fluorescence microscopy but their enzymatic properties were not investigated.

Compound **5** was originally designed to study undecaprenyl pyrophosphate synthetase (UPPs) which produces C₅₅ undecaprenyl pyrophosphate (UPP) via consecutive elongation reactions using eight IPP units with an FPP primer.⁴⁰ In aqueous solution, compound **5** displays $\lambda_{\text{ex}} = 336$ nm and $\lambda_{\text{em}} = 460$ nm. Due to the electron-withdrawing ability of the CF₃ substituent, the lowest unoccupied molecular orbital (LUMO) is decreased compared to the CH₃ counterpart resulting in a red shift of both λ_{ex} and λ_{em} . Compound **5** is both an inhibitor for UPPs when FPP is present and a slow substrate for UPPs when FPP is absent. The K_M of compound **5** (0.69 μ M) and its K_I (0.57 μ M) are similar to the K_M of FPP (0.40 μ M) but the reaction using **5** is significantly slower compared to the reaction using FPP (k_{cat} using **5** is 125-fold less than that obtained using FPP). Similarly, Distefano and coworkers found that compound **5** is a slow substrate for PFTase potentially because of the greater steric bulk of compound **5** relative to FPP.⁴¹ The molecular volume of **5** is approximately

25% larger than FPP. The $k_{\text{cat}}/K_{\text{M}}$ value for compound **5** is more than 350-fold lower than that for FPP. However, it was shown that if the active site of PFTase was expanded by mutating Tyr at 205 to Ala, the mutant enzyme can use compound **5** much more efficiently ($k_{\text{cat}}/K_{\text{M}}$ increases approximately 300-fold compared to wild-type enzyme, Table 1).

In the design of fluorescent isoprenoid analogues, the fluorophore should be small to mimic the natural substrate but the fluorescent intensity should also be high to provide maximal sensitivity. The fluorophore 7-nitro-benzo[1,2,5]oxadiazol-4-ylamino (NBD) is example that achieves a good balance between minimal size and maximal fluorescence. The FPP analogue (**6a**) and GGPP analogue (**6b**) have $\lambda_{\text{ex}} = 487 \text{ nm}$ and $\lambda_{\text{em}} = 550 \text{ nm}$.⁴² Addition of excess PFTase, GGTase-I or GGTase-II to a solution of **6a** or **6b** resulted in large fluorescent changes allowing dissociation constants of 1.6 nM, 6.0 nM and 328 nM for PFTase, GGTase-I and GGTase-II, respectively, to be determined by fluorescence titration (Table 1).

A second useful feature of appending a fluorophore onto the isoprenoid chain is that it facilitates the visualization of prenylated proteins if the isoprenoid analogue is an alternative substrate. In an *in vitro* prenylation assay, prenylated proteins can be resolved via SDS-PAGE and fluorescence intensity can be quantified as exemplified

with **2a**, **2b**, **6a** and **6b**. If the sensitivity of the assay is high enough, steady-state kinetics can be measured as in the case of **6a** and **6b** (Table 1). In cell labeling experiments, *in vivo* conjugation can be imaged if the isoprenoid analogue is stable. Transiently expressed YFP-K-Ras and CFP-RabGDI in COS-7 cells in the presence of **6a** or **6b** were successfully visualized after analog incorporation using SDS-PAGE analysis; the intracellular distribution of compound **6b** in human epidermal carcinoma A431 cells was also visualized via fluorescence microscopy.⁴²

BODIPY-labeled derivatives **7a** and **7b** displayed large fluorescence changes upon binding to protein prenyltransferases, which allowed for the determination of their affinities using fluorescence titration experiments.⁴³ The results showed that **7a** ($K_D = 1.1$ nM), **7b** ($K_D = 4.9$ nM), and FPP ($K_D = 28-46$ nM) have high affinities for mPFTase and **7a** ($K_D = 0.25$ nM) and GGPP ($K_D = 0.23$ nM) have comparable affinities for GGTase-I. However, GGTase-II displayed an affinity for **7a** ($K_D = 245$ nM) substantially lower compared to FPP ($K_D = 60$ nM). Unfortunately among the three enzymes, only GGTase-II can utilize **7a** as a substrate to prenylate its protein substrates whereas PFTase and GGTase-I could not. This highlights the problem of using larger fluorophores that sometimes results in the generation of poor substrates. Time-resolved changes in polarization of the BODIPY fluorophore were used to assay GGTase-II-mediated transfer of **7a** onto Rab 7 and determine a K_M of 200 nM for the

Compound	λ_{ex} (nm)	λ_{ex} (nm)	Substrate (S) or Inhibitor (I)	Peptide or protein substrate	K_M , K_I or K_D {enzyme}
FPP	ND	ND	S	DsGCVLS	$K_M = 27 \mu\text{M}$ {yPFTase} ³⁶
				DsGCVLS	$K_M = 46 \text{ nM}^{44}$, 28 nM^{45} or $1.5 \mu\text{M}^{46}$ {mPFTase}
					$K_D = 60 \text{ nM}$ {mGGTase-II} ³⁸
GGPP	ND	ND	S	DsGCVLL	$K_M = 3.45 \mu\text{M}$ {yGGTase-I} ³⁵
					$K_D = 0.25 \text{ nM}$ {mGGTase-I} ⁴²
					$K_D = 8 \text{ nM}$ {mGGTase-II} ³⁸
1a	360	465	I	DsGCVLL	$K_I = 8.8 \mu\text{M}$ {yPFTase} ³⁶
1b	310	410	S	DsGCVLL	$K_M = 0.33 \mu\text{M}$ {yGGTase-I} ³⁵
2a	340	435	S		$K_D = 330 \text{ nM}$ {mGGTase-II} ³⁸
2b	340	435	S		$K_D = 49 \text{ nM}$ {mGGTase-II} ³⁸
3	340	422	I		$\text{IC}_{50} = \sim 50 \text{ nM}$ {mPFTase} ³⁹
5	336	460	S	GFP-CVIA	$K_M = 1.4 \mu\text{M}$, $k_{\text{cat}} = 1.3 \times 10^{-3} \text{ s}^{-1}$ {mPFTase (WT)} ⁴¹
				GFP-CVIA	$K_M = 0.12 \mu\text{M}$, $k_{\text{cat}} = 0.035 \text{ s}^{-1}$ {mPFTase (Y205A)} ⁴¹
6a	487	550	S	K-Ras	$K_D = 1.6 \text{ nM}$ $K_M = 0.5 \mu\text{M}$, $k_{\text{cat}} = 0.09 \text{ s}^{-1}$ {mPFTase} ⁴²
6b	487	550	S	RhoA	$K_D = 6 \text{ nM}$, $K_M = 0.03 \mu\text{M}$, $k_{\text{cat}} = 0.08 \text{ s}^{-1}$ {mGGTase-I} ⁴²
				Rab7	$K_D = 328 \text{ nM}$, $K_M = 4.7 \mu\text{M}$, $k_{\text{cat}} = 0.04 \text{ s}^{-1}$ {mGGTase-II} ⁴²
7a	490	505	I		$K_D = 1.1 \text{ nM}$ {mPFTase} ⁴³
			I		$K_D = 0.23 \text{ nM}$ {mGGTase-I} ⁴³
			S	Rab7	$K_D = 245 \text{ nM}$; $K_M = 200 \text{ nM}$ {mGGTase-II} ⁴³
7b	490	505	I		$K_D = 4.9 \text{ nM}$ {mPFTase} ⁴³

Table 1. Summary of properties of fluorescent isoprenoid analogues used to study protein prenylation.

isoprenoid substrate; while no k_{cat} value was reported, the authors did note that the rate obtained for GGTase-II-catalyzed prenylation of Rab7 with **7a** was comparable to that observed with **6b** suggesting that the former is an efficient substrate for GGTase-II.⁴³ Finally, compound **7a** was also used to monitor its localization in baby hamster kidney (BHK) cells and in whole zebrafish, demonstrating the utility of these fluorescent compounds for imaging applications.

Probes for prenylomic analysis

Despite the fact that protein prenylation is a widespread phenomenon in eukaryotic cells that is essential for the proper function of many important signaling proteins, the total number of prenylated proteins which comprise the prenylome is still unclear. Only a fraction of predicted prenylated proteins have been experimentally confirmed, leaving the true size of the eukaryotic prenylome unknown. Prenylated proteins have been traditionally detected by metabolic labeling with radioactive substrates such as [³H]mevalonate, [³H]FPP or [³H]GGPP.⁴⁷ However, this method suffers from disadvantages including low sensitivity and long exposure times necessary for detection (weeks to months). To address these limitations, chemical probes for protein prenylation have been developed to improve the detection of prenylated proteins and to allow for their selective enrichment (Figure 4); as was

noted above, these analogues generally involve modification of the third isoprenoid unit proximal to C-12 of FPP (Figure 1, Lower Panel, C-12 in colored in cyan). These molecules differ from the fluorescent analogues **1-7** described above because they contain groups suitable either for pull-down, immunological detection, or further chemical functionalization through bioorthogonal reaction; while the fluorescent analogues discussed above are useful for labeling experiments performed with purified proteins *in vitro*, the fluorophores generally lack the sensitivity necessary to allow visualization of prenylated proteins in cellular extracts.

Figure 4. Synthetic probes for studying the prenylome.

The biotinylated derivative **8b** allowed prenylated proteins to be enriched by pull-down with streptavidin beads and subsequent analysis by Western blot and MS.⁴⁸ While the affinity of compound **8b** for PFTase, GGTase-I and GGTase-II is reduced compared to their native substrates it is comparable to that of the isoprenoid analogues noted above. Unfortunately, compound **8b** is only a substrate for wild type GGTase-II. Little or no transfer activity could be detected for PFTase and GGTase-I. While such selectivity is helpful for labeling only substrates of GGTase-II, it makes the probe less useful for examining the entire prenylome. Mutants of the PFTase (W102T_Y154T or W102T_Y154T_Y205T) and GGTase-I (F52Y_F53Y_Y126T) with expanded active sites were found to use compound **8b** as a substrate allowing the detection of prenylated protein substrates. However, there are two disadvantages of using compound **8b** as a probe to detect prenylated proteins. First, if PFTase or GGTase substrates are being sought, cells must be transfected to express the mutant transferase enzymes. This adds an extra complication and limits its applicability to *ex vivo* (cell culture) systems. Moreover, using transfected cells may not faithfully reproduce the actual physiologically relevant levels of prenyltransferases found in normal cells. Second, it is known from crystallography that in the active sites of both

PFTase and GGTase-I, the protein and isoprenoid substrates physically contact each other⁴⁹ so that the structure of the isoprenoid can influence the peptide specificity. Thus, the identified prenylome may not be the true native prenylome if the isoprenoid analogues differ significantly in structure relative to FPP or GGPP; this is a subtle but important pitfall. Efforts to truncate the structure to create an alternative substrate for PFTase and GGTase-I were not successful. A biotinylated derivative with shortened chain length (**8a**) was again transferred only by GGTase-II and was transferred less efficiently than compound **8b** suggesting that shortening of the isoprenoid chain resulted in a reduction in the affinity for the enzyme.⁴³

A solution to the above mentioned problems is to use isoprenoid analogues with smaller functional handles that are bona fide alternative substrates. After prenylation, a capture/labeling reagent can be attached via a bioorthogonal chemical reaction. The choice of ligation chemistry depends on the downstream application with the common ones being the ‘click’ reaction (also known as the Cu-catalyzed alkyne-azide cycloaddition, CuAAC reaction)⁵⁰ and Staudinger reaction.⁵¹ The advantage of this strategy is that different types of chemical handles can be ligated with one kind of isoprenoid analogue. A number of such reagents including affinity labels (e.g., biotin, FLAG, etc.), reporter dyes and oligonucleotide tags are commercially available. Isoprenoids of this kind include compounds **9a**, **9b**, **10a**, **10b**, **11a** and **11b**. All of

these analogues are substrates for PFTase.

Growth of COS cells in the presence of compound **9a** or its unphosphorylated alcohol version resulted in incorporation of this azide-containing isoprenoid into proteins. Chemoselective derivatization of prenylated proteins by a Staudinger reaction was accomplished using a biotinylated phosphine capture reagent.⁵² The resulting protein conjugates were specifically detected or affinity purified using streptavidin-linked horseradish peroxidase or agarose beads, respectively. Subsequent MS analysis identified 21 different farnesylated proteins including those with potentially novel farnesylation motifs. It was reported that the azide of compound **9a** can isomerize between C-12 and C-10 yielding a mixture of products that can complicate analysis.⁵³ Use of compound **9b** should overcome this problem.²³ Similar experiments with **9c**, a geranylgeranyl diphosphate analogue allowed 10 different novel geranylgeranylated proteins to be identified including Rap2c.⁵⁴ Tate and coworkers used the same analogue in conjunction with a multifunctional labeling reagent that incorporated biotin and rhodamine to successfully enrich and visualize Rab proteins.⁵⁵

The rapid rate of the CuAAC reaction has made it the method of choice for many proteomic profiling protocols as an alternative to the Staudinger reaction. However, background labeling occurs in that reaction when the alkyne reagent is used in

excess.^{56,57} Significantly lower levels of non-specific reaction occur when the azide partner is employed at high concentration. DeGraw et al. compared the labeling of prenylated proteins with TAMRA-based detection reagents in HeLa cells after metabolic labeling with either compound **10a** or the alcohol form of **9a**. SDS-PAGE followed by in-gel fluorescence analysis showed that incorporation using compound **10a** followed by detection with TAMRA-azide gave significantly lower levels of background labeling relative to experiments employing **9a** and TAMRA-alkyne.⁵⁶ 2-D gel electrophoresis of labeled proteins from HeLa cells grown in the presence of the unphosphorylated form of **10a** followed by MS analysis allowed 7 different modified proteins to be identified. A differential gel electrophoresis-based method using **6b** in which the levels of prenylated proteins in the presence and absence of a farnesylation inhibitor were compared allowed the identification of proteins whose levels both decreased (8 proteins) and increased (11 proteins) in response to the drug.⁵⁸ These results are particularly noteworthy since they demonstrate how cellular physiology in response to therapeutic agents can be analyzed using these types of experiments.

Hang and coworkers have compared the labeling signals and patterns using alkyne-containing probes of varying size.⁵⁷ Jurkat cells were treated with the free alcohol forms of **9a**, **10a**, **11a** and **12a** and the resulting cell lysates were conjugated via the CuAAC reaction with rhodamine-azide or rhodamine-alkyne. Lysates were

separated by SDS-PAGE and scanned for fluorescence. Much better labeling compared with background was obtained using **10a**, **11a** and **12a** compared to **9a**. This is consistent with other reports concerning the use of azide- versus alkyne-based probes.⁵⁶ Labeling was stronger with **10a** and **11b** compared with **9a** and **11a**. In that work, **10a** was chosen for subsequent experiments because its synthesis is the most straightforward and was found to be more stable compared to other alkyne-containing isoprenoid probes.^{57,59} Interestingly, they used **10a** to show that SifA, a protein from *S. typhimurium* involved in bacterial replication was prenylated in HeLa cells. That result was particularly noteworthy since it suggests that the bacteria rely on host prenyltransferases to produce prenylated proteins important for bacterial replication. In a subsequent paper, they extended these results using an improved capture and release azide-based reagent to identify a large number of putative prenylated proteins and demonstrated that prenylation of the long isoform of ZAP was essential for its membrane targeting and antiviral activity.⁴³ In addition to the azide- and alkyne-modified isoprenoid analogues described above, probes containing aldehydes,²⁶ ketones, strained alkenes⁶⁰ and multiple functional groups²⁷ that provide additional levels of chemical orthogonality have also been prepared; these may be useful for the next generation of metabolic labeling experiments since they could allow multiple modifications to be analyzed simultaneously.

Finally, it should be noted that antibodies have proven to be useful in both the detection and immunoprecipitation of proteins with post-translational modifications. However, it was reported that antibodies produced to detect farnesylated proteins showed cross-reaction with other proteins.⁶¹⁻⁶³ An alternative approach to detect prenylated proteins is to produce antibodies against polypeptides modified with non-natural farnesyl analogues. In this way, it is less likely that the resulting antibodies will manifest crossreactivity. Since compound **12** is also an effective alternative substrate for PFTase, Spielman and coworkers have produced antibodies specific for the anilino geranyl moiety.³⁴ The alcohol version of compound **12** was incorporated into cellular proteins in a PFTase dependent manner. In a subsequent report, 2-D electrophoresis and western blotting with these antibodies allowed the detection of numerous farnesylated proteins in the proteome of leukemia cells.⁶⁴

Concluding Remarks and Future Directions

The importance of protein prenylation for functional biochemical signaling has stimulated considerable interest in this post-translational modification and synthetic isoprenoid analogues have proved to be quite useful for studying this process. Compounds that incorporate fluorescent moieties have allowed the binding affinities of isoprenoids to prenylating enzymes to be quantified; they have also facilitated the

preparation of fluorescently labeled forms of prenylated proteins for detection in various assays. Synthetic isoprenoid probes incorporating biotin, bio-orthogonal functional groups or antigenic moieties have allowed prenylated proteins present in cell culture to be identified; this includes known proteins as well as several previously uncharacterized ones. Despite this success, there is still considerable work to be done. While over 600 putative prenylation sequences are known in the human genome, only about 50 have been conclusively identified; methods capable of more sensitive detection are needed to address this. In addition, to date, all proteomic experiments have been performed in cell culture. These tools have yet been generally applied in tissue samples or whole animal models to study disease. Highly efficient incorporation coupled with sensitive detection will be necessary to accomplish that goal. Finally, it should be noted that the enzymatic alkylation of cysteine residues with isoprenoids is only the first step in the biogenesis of mature prenylated proteins. Additional enzymatic steps including proteolysis, methylation and fatty acid acylation are necessary in certain cases to produce fully functional proteins. Synthetic isoprenoid probes should prove to be highly useful for the study of those processes as well. Recently, simple methods have been developed for the synthesis of prenylated peptides bearing C-terminal methyl esters.^{65,66} This should facilitate studies of these later steps.

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References

- (1) Bos, J. L. *Cancer Res.* **1989**, *49*, 4682.
- (2) Saxena, N.; Lahiri, S. S.; Hambarde, S.; Tripathi, R. P. *Cancer Invest.* **2008**, *26*, 948.
- (3) Lackner, M. R.; Kindt, R. M.; Carroll, P. M.; Brown, K.; Cancilla, M. R.; Chen, C.; de Silva, H.; Franke, Y.; Guan, B.; Heuer, T.; Hung, T.; Keegan, K.; Lee, J. M.; Manne, V.; O'Brien, C.; Parry, D.; Perez-Villar, J. J.; Reddy, R. K.; Xiao, H.; Zhan, H.; Cockett, M.; Plowman, G.; Fitzgerald, K.; Costa, M.; Ross-Macdonald, P. *Cancer Cell* **2005**, *7*, 325.
- (4) Long, S. B.; Casey, P. J.; Beese, L. S. *Structure*, *8*, 209.
- (5) Moores, S. L.; Schaber, M. D.; Mosser, S. D.; Rands, E.; O'Hara, M. B.; Garsky, V. M.; Marshall, M. S.; Pompiano, D. L.; Gibbs, J. B. *J. Biol. Chem.* **1991**, *266*, 14603.
- (6) Fu, H. W., and Casey, P. J. *Recent Prog. Horm. Res.* **1999**, *54*, 315.
- (7) Wang, Y.-C.; Dozier, J. K.; Beese, L. S.; Distefano, M. D. *ACS Chem. Biol.* **2014**, *9*, 1726.
- (8) Rak, A.; Pylypenko, O.; Niculae, A.; Pyatkov, K.; Goody, R. S.; Alexandrov, K. *Cell* **2004**, *117*, 749.
- (9) Winter-Vann, A. M.; Casey, P. J. *Nat. Rev. Cancer* **2005**, *5*, 405.
- (10) Epstein, W. W.; Lever, D.; Leining, L. M.; Bruenger, E.; Rilling, H. C. *Proc. Natl. Acad. Sci. USA.* **1991**, *88*, 9668.
- (11) Roskoski Jr, R. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 1.
- (12) Horiuchi, H.; Kawata, M.; Katayama, M.; Yoshida, Y.; Musha, T.; Ando, S.; Takai, Y. *J. Biol. Chem.* **1991**, *266*, 16981.
- (13) Song, J. L.; White, T. C. *Microbiology* **2003**, *149*, 249.
- (14) Chakrabarti*, D.; Azam, T.; DelVecchio, C.; Qiu, L.; Park, Y.-i.; Allen, C. M. *Mol. Biochem. Parasit.* **1998**, *94*, 175.
- (15) Gelb, M. H.; Brunsveld, L.; Hrycyna, C. A.; Michaelis, S.; Tamanoi, F.; Van Voorhis, W. C.; Waldmann, H. *Nat. Chem. Biol.* **2006**, *2*, 518.
- (16) Hast, M. A.; Fletcher, S.; Cummings, C. G.; Pusateri, E. E.; Blaskovich, M. A.; Rivas, K.; Gelb, M. H.; Van Voorhis, W. C.; Sebt, S. M.; Hamilton, A. D.; Beese, L. S. *Chem. Biol.* **2009**, *16*, 181.
- (17) Hast, M. A.; Nichols, C. B.; Armstrong, S. M.; Kelly, S. M.; Hellings, H. W.; Alspaugh, J. A.; Beese, L. S. *J. Biol. Chem.* **2011**, *286*, 35149.
- (18) Sunami, S.; Ohkubo, M.; Sagara, T.; Ono, J.; Asahi, S.; Koito, S.; Morishima, H. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 629.
- (19) Berndt, N.; Hamilton, A. D.; Sebt, S. M. *Nat. Rev. Cancer* **2011**, *11*, 775.
- (20) Palsuledesai, C. C.; Distefano, M. D. *ACS Chem. Biol.* **2015**, *10*, 51.

- (21) Randall, S. K.; Crowell, D. N. *Crit. Rev. Biochem. Mol.* **1999**, *34*, 325.
- (22) Hemsley, P. A. *New Phytol.* **2015**, *205*, 476.
- (23) Duckworth, B. P.; Xu, J.; Taton, T. A.; Guo, A.; Distefano, M. D. *Bioconjugate Chem.* **2006**, *17*, 967.
- (24) Gauchet, C.; Labadie, G. R.; Poulter, C. D. *J. Am. Chem. Soc.* **2006**, *128*, 9274.
- (25) Ochocki, J. D.; Distefano, M. D. *Med. Chem. Commun.* **2013**, *4*, 476.
- (26) Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D. *J. Am. Chem. Soc.* **2012**, *134*, 8455.
- (27) Rashidian, M.; Kumarapperuma, S. C.; Gabrielse, K.; Fegan, A.; Wagner, C. R.; Distefano, M. D. *J. Am. Chem. Soc.* **2013**, *135*, 16388.
- (28) Duckworth, B. P.; Chen, Y.; Wollack, J. W.; Sham, Y.; Mueller, J. D.; Taton, T. A.; Distefano, M. D. *Angew. Chem. Int. Ed.* **2007**, *46*, 8819.
- (29) Yeo, J. E.; Wickramaratne, S.; Khatwani, S.; Wang, Y.-C.; Vervacke, J.; Distefano, M. D.; Tretyakova, N. Y. *ACS Chem. Biol.* **2014**, *9*, 1860.
- (30) Kale TA, H. S., Rose MW, Distefano MD. *Curr. Top. Med. Chem.* **2003**, *3*, 1043.
- (31) Vervacke JS, W. Y., Distefano MD. *Curr. Med. Chem.* **2013**, *20*, 1585.
- (32) Gibbs, R. A. Z., T. J.; Sebolt-Leopold, J. S. *Curr. Med. Chem.* **2001**, *8*, 1437.
- (33) Chen, M.; Knifley, T.; Subramanian, T.; Spielmann, H. P.; O'Connor, K. L. *PLoS One* **2014**, *9*, e89892.
- (34) Troutman, J. M.; Roberts, M. J.; Andres, D. A.; Spielmann, H. P. *Bioconjugate Chem.* **2005**, *16*, 1209.
- (35) Liu, X.-h.; Prestwich, G. D. *J. Am. Chem. Soc.* **2002**, *124*, 20.
- (36) Liu, X.-h.; Prestwich, G. D. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2137.
- (37) Owen, D. J.; Alexandrov, K.; Rostkova, E.; Scheidig, A. J.; Goody, R. S.; Waldmann, H. *Angew. Chem. Int. Ed.* **1999**, *38*, 509.
- (38) Thomä, N. H.; Iakovenko, A.; Owen, D.; Scheidig, A. S.; Waldmann, H.; Goody, R. S.; Alexandrov, K. *Biochemistry* **2000**, *39*, 12043.
- (39) Kim; Kleckley, T. S.; Wiemer, A. J.; Holstein, S. A.; Hohl, R. J.; Wiemer, D. F. *J. Org. Chem.* **2004**, *69*, 8186.
- (40) Chen, A. P. C.; Chen, Y.-H.; Liu, H.-P.; Li, Y.-C.; Chen, C.-T.; Liang, P.-H. *J. Am. Chem. Soc.* **2002**, *124*, 15217.
- (41) Dozier, J. K.; Khatwani, S. L.; Wollack, J. W.; Wang, Y.-C.; Schmidt-Dannert, C.; Distefano, M. D. *Bioconjugate Chem.* **2014**, *25*, 1203.
- (42) Dursina, B.; Reents, R.; Delon, C.; Wu, Y.; Kulharia, M.; Thutewohl, M.; Veligodsky, A.; Kalinin, A.; Evstifeev, V.; Ciobanu, D.; Szedlacsek, S. E.; Waldmann, H.; Goody, R. S.; Alexandrov, K. *J. Am. Chem. Soc.* **2006**, *128*, 2822.
- (43) Das, D.; Tnimov, Z.; Nguyen, U. T. T.; Thimmaiah, G.; Lo, H.; Abankwa, D.; Wu, Y.; Goody, R. S.; Waldmann, H.; Alexandrov, K. *ChemBioChem* **2012**, *13*, 674.

- (44) Chehade, K. A. H.; Kiegiel, K.; Isaacs, R. J.; Pickett, J. S.; Bowers, K. E.; Fierke, C. A.; Andres, D. A.; Spielmann, H. P. *J. Am. Chem. Soc.* **2002**, *124*, 8206.
- (45) Rawat, D. S.; Krzysiak, A. J.; Gibbs, R. A. *J. Org. Chem.* **2008**, *73*, 1881.
- (46) Subramanian, T.; Pais, J. E.; Liu, S.; Troutman, J. M.; Suzuki, Y.; Leela Subramanian, K.; Fierke, C. A.; Andres, D. A.; Spielmann, H. P. *Biochemistry* **2012**, *51*, 8307.
- (47) Maltese, W. A. *The FASEB J.* **1990**, *4*, 3319.
- (48) Nguyen, U. T. T.; Guo, Z.; Delon, C.; Wu, Y.; Deraeve, C.; Franzel, B.; Bon, R. S.; Blankenfeldt, W.; Goody, R. S.; Waldmann, H.; Wolters, D.; Alexandrov, K. *Nat. Chem. Biol.* **2009**, *5*, 227.
- (49) Scott Reid, T.; Terry, K. L.; Casey, P. J.; Beese, L. S. *J. Mol. Biol.* **2004**, *343*, 417.
- (50) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192.
- (51) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007.
- (52) Kho, Y.; Kim, S. C.; Jiang, C.; Barma, D.; Kwon, S. W.; Cheng, J.; Jaunbergs, J.; Weinbaum, C.; Tamanoi, F.; Falck, J.; Zhao, Y. *Proc. Natl. Acad. Sci. USA.* **2004**, *101*, 12479.
- (53) Rose, M. W.; Rose, N. D.; Boggs, J.; Lenevich, S.; Xu, J.; Barany, G.; Distefano, M. D. *J. Pept. Res.* **2005**, *65*, 529.
- (54) Chan, L. N.; Hart, C.; Guo, L.; Nyberg, T.; Davies, B. S. J.; Fong, L. G.; Young, S. G.; Agnew, B. J.; Tamanoi, F. *Electrophoresis* **2009**, *30*, 3598.
- (55) Berry, A. F. H.; Heal, W. P.; Tarafder, A. K.; Tolmachova, T.; Baron, R. A.; Seabra, M. C.; Tate, E. W. *ChemBioChem* **2010**, *11*, 771.
- (56) DeGraw, A. J.; Palsuledesai, C.; Ochocki, J. D.; Dozier, J. K.; Lenevich, S.; Rashidian, M.; Distefano, M. D. *Chem. Biol. Drug Des.* **2010**, *76*, 460.
- (57) Charron, G.; Tsou, L. K.; Maguire, W.; Yount, J. S.; Hang, H. C. *Mol. BioSyst.* **2011**, *7*, 67.
- (58) Palsuledesai, C. C.; Ochocki, J. D.; Markowski, T. W.; Distefano, M. D. *Mol. BioSyst.* **2014**, *10*, 1094.
- (59) Charron, G.; Li, M. M. H.; MacDonald, M. R.; Hang, H. C. *Proc. Natl. Acad. Sci. USA.* **2013**, *110*, 11085.
- (60) Wollack, J. W.; Monson, B. J.; Dozier, J. K.; Dalluge, J. J.; Poss, K.; Hilderbrand, S. A.; Distefano, M. D. *Chem. Biol. Drug Des.* **2014**, *84*, 140.
- (61) Liu, X.-h.; Suh, D.-Y.; Call, J.; Prestwich, G. D. *Bioconjugate Chem.* **2004**, *15*, 270.
- (62) Lin, H. P.; Hsu, S. C.; Wu, J. C.; Sheen, I. J.; Yan, B. S.; Syu, W. J. *J. Gen. Virol.* **1999**, *80*, 91.

- (63) Baron, R.; Fourcade, E.; Lajoie-Mazenc, I.; Allal, C.; Couderc, B.; Barbaras, R.; Favre, G.; Faye, J.-C.; Pradines, A. *Proc. Natl. Acad. Sci. USA*. **2000**, *97*, 11626.
- (64) Onono, F. O.; Morgan, M. A.; Spielmann, H. P.; Andres, D. A.; Subramanian, T.; Ganser, A.; Reuter, C. W. M. *Mol. Cell. Proteomics* **2010**, *9*, 742.
- (65) Diaz-Rodriguez, V.; Mullen, D. G.; Ganusova, E.; Becker, J. M.; Distefano, M. D. *Org. Lett.* **2012**, *14*, 5648.
- (66) Diaz-Rodriguez, V.; Ganusova, E.; Rappe, T. M.; Becker, J. M.; Distefano, M. D. *J. Org. Chem.* **2015**, *80*, 11266.

Graphical Abstract