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a-Factor Analogues Containing Alkyne- and Azide-Functionalized Isoprenoids Are Efficiently Enzymatically Processed and Retain Wild-Type Bioactivity

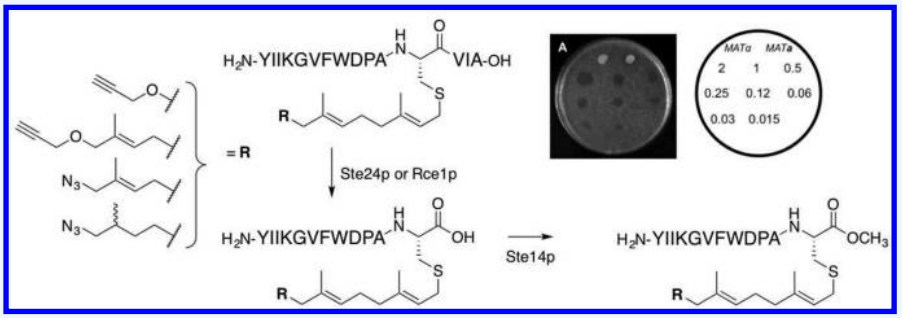
Veronica Diaz-Rodriguez,[†] Erh-Ting Hsu,[‡] Elena Ganusova,[§] Elena R. Werst,[†] Jeffrey M. Becker,[§] Christine A. Hrycyna,[‡] and Mark D. Distefano*[,†]

[†]Department of Chemistry, University of Minnesota, 207 Pleasant Street SE, Minneapolis, Minnesota 55455, United States

[‡]Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, United States

[§]Department of Microbiology, University of Tennessee, Circle Park Drive, Knoxville, Tennessee 37996, United States

Supporting Information



ABSTRACT: Protein prenylation is a post-translational modification that involves the addition of one or two isoprenoid groups to the C-terminus of selected proteins using either farnesyl diphosphate or geranylgeranyl diphosphate. Three crucial enzymatic steps are involved in the processing of prenylated proteins to yield the final mature product. The farnesylated dodecapeptide, **a**-factor, is particularly useful for studies of protein prenylation because it requires the identical three-step process to generate the same C-terminal farnesylated cysteine methyl ester substructure present in larger farnesylated proteins. Recently, several groups have developed isoprenoid analogs bearing azide and alkyne groups that can be used in metabolic labeling experiments. Those compounds have proven useful for profiling prenylated proteins and also show great promise as tools to study how the levels of prenylated proteins vary in different disease models. Herein, we describe the preparation and use of prenylated **a**-factor analogs, and precursor peptides, to investigate two key questions. First, **a**-factor analogues containing modified isoprenoids were prepared to evaluate whether the non-natural lipid group interferes with the biological activity of the **a**-factor. Second, **a**-factor-derived precursor peptides were synthesized to evaluate whether they can be efficiently processed by the yeast proteases Rce1 and Ste24 as well as the yeast methyltransferase Ste14 to yield mature **a**-factor analogues. Taken together, the results reported here indicate that metabolic labeling experiments with azide- and alkyne-functionalized isoprenoids can yield prenylated products that are fully processed and biologically functional. Overall, these observations suggest that the isoprenoids studied here that incorporate bioorthogonal functionality can be used in metabolic labeling experiments without concern that they will induce undesired physiological changes that may complicate data interpretation.

INTRODUCTION

Protein prenylation is a post-translational modification that involves the addition of one or two isoprenoid groups to the C-terminus of selected proteins using either farnesyl diphosphate (C_{15}, FPP, **1a**) or geranylgeranyl diphosphate (C_{20}, GGPP).[1] Three crucial steps are involved in the maturation of prenylated proteins as is illustrated for farnesylation in Figure 1. First, prenylation of a cysteine residue occurs in a reaction catalyzed by one of three prenyltransferases. Two of those enzymes catalyze the addition of a single isoprenoid group to proteins containing a C-terminal CaaX box sequence where C is a cysteine residue, a is an aliphatic residue, and X is an amino acid that determines whether a farnesyl or geranylgeranyl group is attached;[2−4] a third enzyme promotes the transfer of two geranylgeranyl groups to C-terminal sequences ending in CC or CXC.[5] The second step involves the removal of the aaX tripeptide from the newly prenylated CaaX-box to yield a protein containing a C-terminal prenylcysteine. This step is catalyzed by Ras converting enzyme (Rce1) or Ste24.[6−8] The final step involves the action of a carboxylmethyltransferase (Ste14), which catalyzes the transfer of a methyl group from S-

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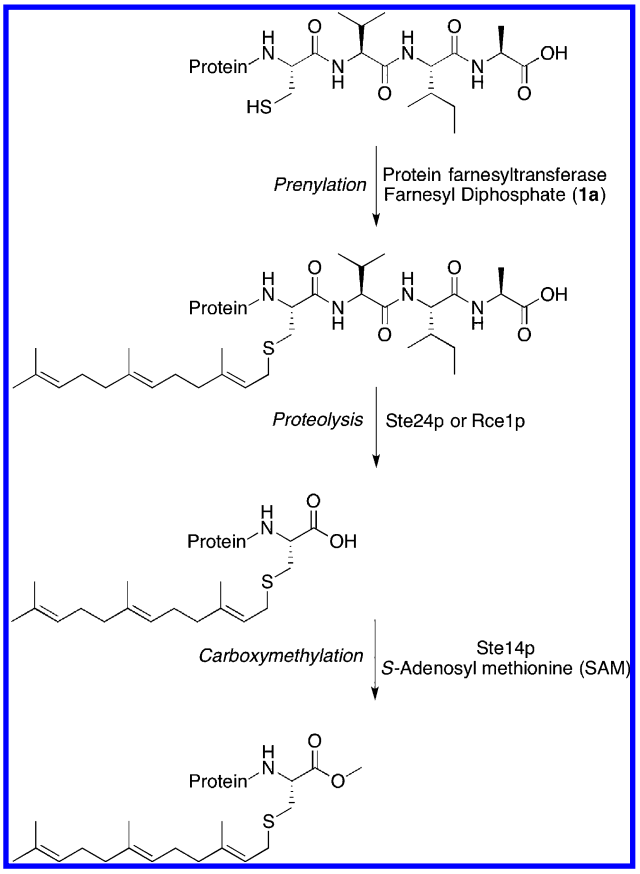

Figure 1. Three-step process for the biogenesis of prenylated proteins illustrated for the farnesylation and subsequent processing of a protein with a C-terminal CVIA CaaX-box sequence.

adenosyl methionine to the carboxylate of the C-terminal prenyl cysteine residue to yield a C-terminal methyl ester.[9]

A large number of proteins are known to be prenylated,[10] and these polypeptides, including many members of the Ras superfamily, often associate with the plasma membrane where they interact with a variety of proteins involved in signal transduction pathways.[11] Those interactions, in turn, control a plethora of cellular processes ranging from cell growth to differentiation.[12] Due to the critical roles that Ras proteins play, inhibitors of protein prenylation have been explored as possible anticancer agents[13] as well as for other therapeutic applications ranging from tropical diseases in protozoa[14] to Alzheimer's disease.[15] It is of particular significance, despite the importance of prenylated proteins in disease, that the full complement of prenylated proteins present in human cells has yet to be elucidated. In addition, how the levels of such proteins vary under different physiological conditions or disease states is also not well understood. Recently, several groups have developed isoprenoid analogs bearing azide, alkyne, and related groups that can be used in metabolic labeling experiments.[16−20] Those compounds have proven useful for profiling prenylated proteins and also show great promise as tools to study how the levels of prenylated proteins vary in different disease models.[21−24] However, at present there is no information about the activity of proteins modified with such groups. Additionally, while *in vitro* enzymological experiments have provided convincing evidence that prenyltransferases can incorporate such analogs,[25,26] there are no reports concerning the ability of the two proteases or the methyltransferase to act on proteins containing these moieties to convert them to their fully processed state.

The farnesylated dodecapeptide **a**-factor from the yeast *S. cerevisiae* is a particularly useful model for studies of protein prenylation because it is post-translationally processed similarly to other CaaX-proteins to contain the same C-terminal farnesylated cysteine methyl ester substructure present in larger farnesylated proteins.[27] Importantly, the biogenesis of yeast **a**-factor proceeds through the same three-step process used for larger prenylated proteins—namely, prenylation, proteolysis, and methylation (Figure 1).[28,29] Moreover, since **a**-factor binds to a G-protein coupled receptor on cells of the opposite mating type of yeast and initiates growth arrest in those cells, the activity of exogenous **a**-factor can be easily assessed in a simple cell-based assay.[30] A number of groups have used this system extensively to investigate the effects of sequence differences[31] and structural variations in the isoprenoid group on **a**-factor biosynthesis and activity.[32,33] Herein, we describe the use of prenylated **a**-factor analogues and precursor peptides to investigate two key questions. First, **a**-factor analogues containing modified isoprenoids were prepared to evaluate whether the non-natural lipid group interferes with the biological activity of **a**-factor. Second, **a**-factor-derived precursor peptides were prepared and used to evaluate whether they can be efficiently processed by the proteases Rce1 and Ste24 as well as the methyltransferase Ste14 to yield mature **a**-factor analogs.

RESULTS AND DISCUSSION

Synthesis of a-Factor Analogs Containing Modified Isoprenoids. To probe the biological activity of **a**-factor analogues containing modified isoprenoids commonly used in metabolic labeling experiments, it was first necessary to prepare the requisite prenylated peptides (Figure 2). In previous work we reported methodology for the synthesis of **a**-factor and related analogs containing modifications within the peptide[34] and the C-terminal methyl ester.[35] That approach used a side chain anchoring strategy developed for the assembly of peptides containing C-terminal cysteine esters using solid phase peptide synthesis (SPPS) conditions. A collection of isoprenoid analogs containing alkyne and azide functionality commonly used in metabolic labeling experiments was selected for this study. The **a**-factor peptide sequence **2a** was synthesized as previously described.[35] In brief, an Fmoc-Cys-OMe residue was coupled onto Trt-Cl resin via its side chain thiol functionality. The resin-bound product, Fmoc-Cys(Trt-resin)-OMe, was then used to initiate synthesis of the **a**-factor sequence using standard Fmoc/HCTU coupling conditions for chain elongation. Cleavage from the resin and deprotection of side chains was accomplished under acidic conditions to afford the desired unmodified peptide (**2a**). For the synthesis of **a**-factor and analogues containing modified isoprenoids, allylic bromides (**1b**−**1f**) were reacted with **2a** in the presence of $Zn(OAc)_2$ to obtain the desired peptides **2b**−**2f**.[36] The peptides were purified by preparative RP-HPLC and their identities were confirmed by ESI-MS analysis.

Biological Analysis of a-Factor Analogs Containing Modified Isoprenoids. With the desired **a**-factor analogs in hand, each peptide was then assayed to evaluate the effects of the non-native isoprenoid on **a**-factor function. Biological activity was evaluated in a yeast growth arrest halo assay, which exploits the fact that *MATα* cells, which express the **a**-factor receptor Ste3p, undergo growth arrest upon binding **a**-factor.[37,38] In this assay, increasing amounts of exogenous **a**-

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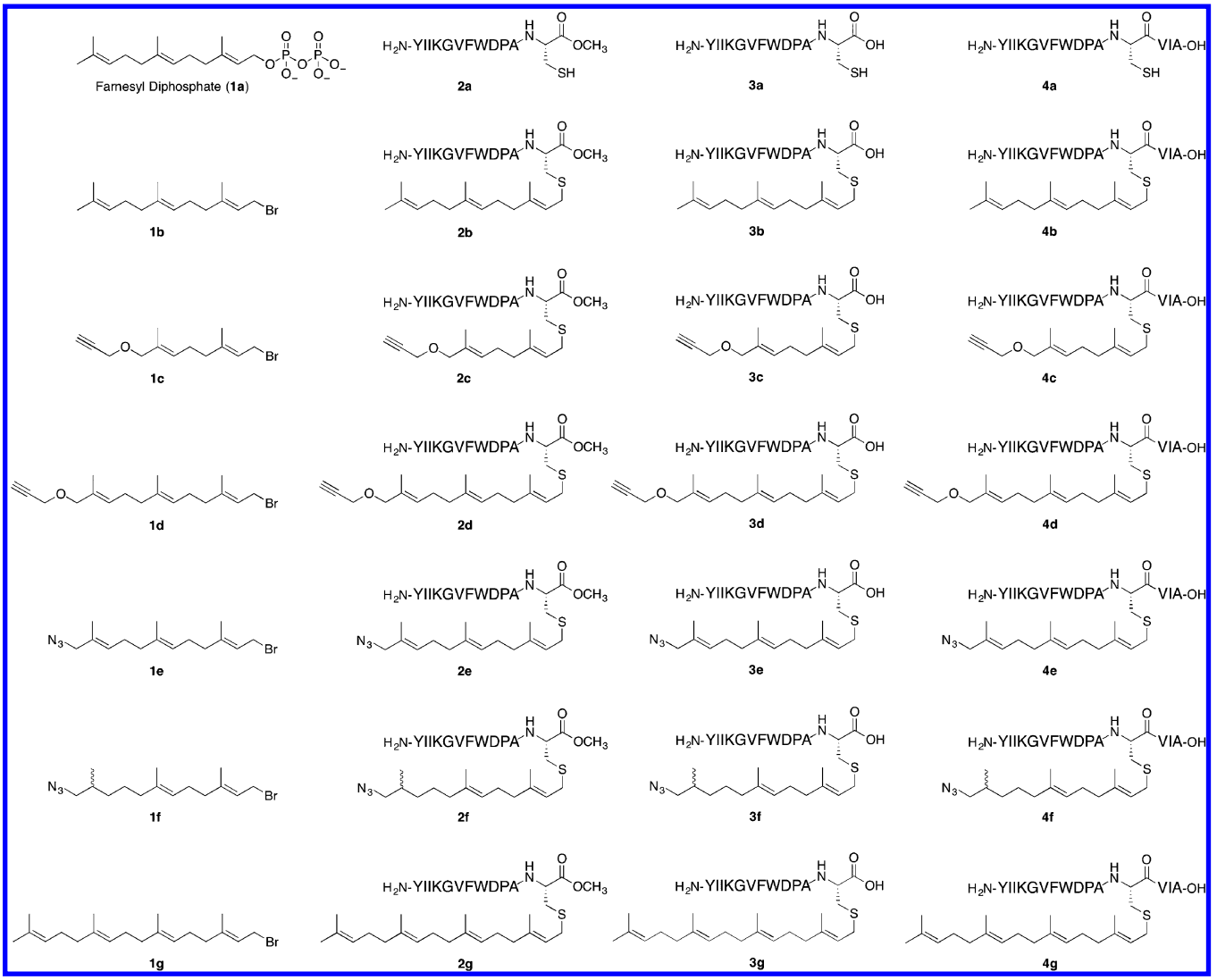

Figure 2. **a**-Factor analogues and precursor peptides incorporating alkyne- and azide-containing isoprenoids. **a**-Factor analogues **2b**−**2f** were prepared by alkylation of **2a** using **1b**−**1f**. **a**-Factor precursors **3b**−**3f** that can serve as substrates for the methyltransferase Ste14 were prepared by alkylation of **3a** with **1b**−**1f**. **a**-Factor precursors **4b**−**4f** that can serve as substrates for the proteases Ste24 and Rce1 to yield **3b**−**3f** were prepared by alkylation of **4a** with **1b**−**1f**. Geranylgeranylated forms of **a**-factor precursors **3g** and **4g** were prepared in an analogous fashion. To facilitate discussion in the text, the isoprenoids derived from **1c**−**1f** are designated as C10Alk, C15Alk, C15Az, and C15dhAz, respectively.

factor or *MAT***a** cells expressing **a**-factor (as a positive control) are spotted onto a plate containing a lawn of *MAT*α RC757 *S. cerevisiae* cells, a strain chosen because of its hypersensitivity to **a**-factor. Growth arrest is visualized as a clear zone of inhibition around the spot added to the lawn. A previously characterized, synthetic **a**-factor with wild-type potency stimulated growth arrest with an end point of 0.12 ng;[39] the synthetic material produced in this study yielded an identical end point (Figure 3, Panel A).

Next, the biological activities of the **a**-factor analogues were evaluated similarly (Figure 3, Panel B, C, D, E). Interestingly, all analogues manifested biological activity that was greater than or equal to that of wild-type (farnesylated) **a**-factor (**2b**). In fact, the biological activity of the C10Alk-modified conjugate (**2c**) was almost an order of magnitude more potent relative to **a**-factor, manifesting an end-point of <0.015 ng; interestingly, Marcus et al. noted that an **a**-factor analogue containing a shorter geranyl group, in place of farnesyl, was also 2-fold more active as compared to the wild-type pheromone.[37] The results presented here for C10Alk, which is also somewhat smaller than farnesyl, are consistent with those earlier observations. The C15Alk-modified conjugate (**2d**) with an end-point of 0.06 ng was 2-fold lower and was also modestly better (2-fold) relative to wild-type **a**-factor; in contrast, the two azide-containing analogues (**2e** and **2f**) manifested the same activity as wild-type **a**-factor. The end-point values for all of the analogues are summarized in Table 1. At present, it is not clear why the alkynes are more potent. While not dramatically dissimilar, they do differ from the azides in terms of both their geometry and polarity that may alter the way they interact with the **a**-factor receptor. As previously noted by Marcus et al, the receptor is quite sensitive to changes in the isoprenoid moiety of **a**-factor.[37] Finally, to ensure that the observed growth arrest was not due to general toxicity, control experiments were performed using LM102 strain (*MAT***a**), which expresses the α-factor receptor and thus does not respond to **a**-factor. *MAT***a** cells did not yield a zone of growth inhibition in response to any of the **a**-factor derivatives indicating that tested compounds are not toxic and are mating type specific (data not shown). Overall, these observations indicate that the alkyne and azide

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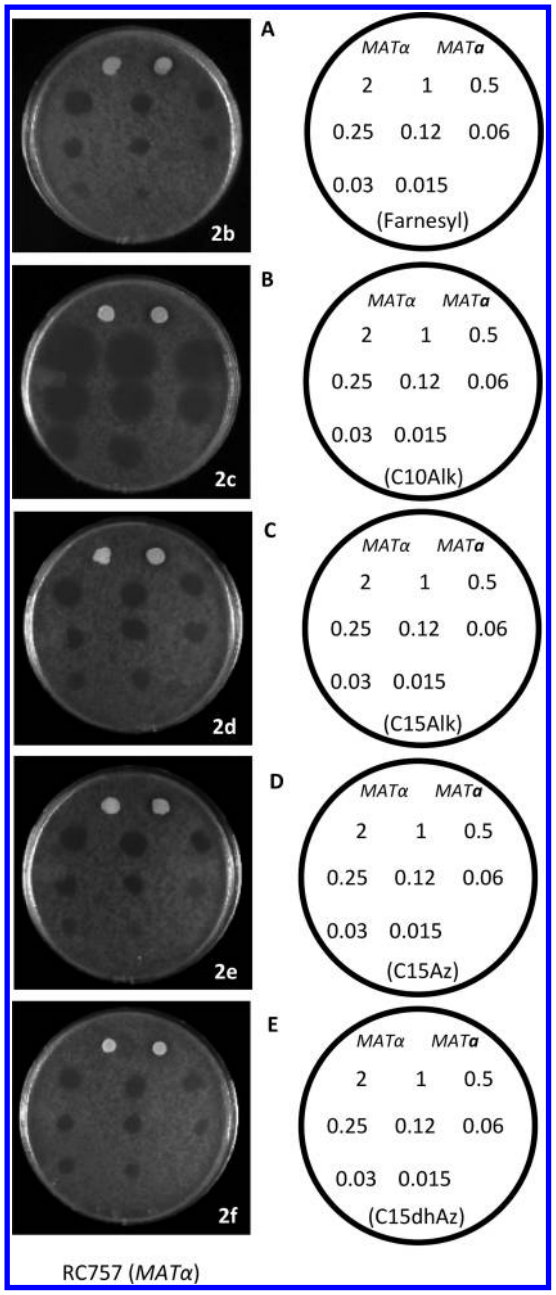

Figure 3. Biological assay of **a**-factor and **a**-factor analogs. Growth arrest in response to **a**-factor or **a**-factor analogues was determined using the **a**-factor responsive strain RC757. A: wild-type **a**-factor (**2b**); B: C10Alk **a**-factor analogue (**2c**); C: C15Alk **a**-factor analogue (**2d**); D: C15Az **a**-factor analogue (**2e**); E: C15dhAz **a**-factor analogue (**2f**). The templates on the right indicate the amount of synthetic compound spotted on plates. At the top of each plate 2.5 μL of cells secreting α-factor (*MATα*, on the left) or **a**-factor (*MATa*, on the right) were applied to the lawn as indicated in the templates. A zone of inhibition formed around the **a**-factor (2–0.015 ng of **a**-factor) secreting cells but not around those secreting α-factor indicating that the RC757 cells respond specifically to **a**-factor.

functionalities incorporated into the isoprenoid moieties do not interfere with the interactions between the modified peptide and the receptor or the resulting signaling that causes the growth arrest of the cells. While these results do not prove that any prenylated protein containing one of these modifications will retain full wild-type activity, they do demonstrate that it is at least possible for these moieties to be completely functional in this highly sensitive biological assay.

Table 1. End-Point Concentrations for a-Factor (2b) and a-Factor Analogs 2c–2g Determined via a Growth Arrest Assay

a-factor analogue	end-point concentration (ng)
2b (WT)	0.12
2c	<0.015
2d	0.06
2e	0.12
2f	0.12
2g	0.10[a]

[a]From Caldwell et al.[40] In that reference, the authors noted that under the conditions the assays were performed, which are slightly different than those used in the work reported here, **2g** manifested only 25% of the activity observed with wild-type **a**-factor (**2b**). The experimental values reported here are the results of four biological replicates.

Synthesis of a-Factor Precursor Peptides Containing Modified Isoprenoids. While the studies described above provide convincing evidence that **a**-factor analogues bearing azide- and alkyne-functionalized isoprenoids with methylated C-termini retain full biological activity in yeast mating, they do not address whether the mature **a**-factor-related peptides containing the modified isoprenoids can actually be produced by the biosynthetic machinery responsible for **a**-factor synthesis. From previous studies, we and others have demonstrated that isoprenoid diphosphates containing azides and alkynes are efficiently incorporated into peptides by farnesyl- and geranylgeranyltransferases *in vitro*. However, the subsequent processing of prenylated peptides incorporating azide- and alkyne-modified isoprenoids by the CaaX-box specific proteases Rce1 and Ste24 and methylation by Ste14 was unexplored. To address this important question, prenylated peptides incorporating the modified isoprenoids were prepared including one series that contained a C-terminal CVIA CaaX-box sequence to examine cleavage by Rce1 or Ste24 and one with a free C-terminal Cys residue to examine methylation by the isoprenylcysteine carboxyl methyltransferase Ste14. The peptides were synthesized using Fmoc/HCTU coupling conditions starting from Fmoc-Cys(Trt)-Wang resin (for the one ending in Cys) and Fmoc-Ala-Wang resin (for the one terminating in CVIA) to produce peptides **3a** and **4a**, respectively, after acidic cleavage/deprotection. These peptides were alkylated in solution as described above using bromides **1b–1g** to produce peptides **3b–3g** and **4b–4g**. Purification and analysis of these peptides was performed by RP-HPLC. The identity of all the peptides was determined by ESI-MS. To confirm the sequence of these peptides that were all prepared from either **3a** or **4a**, MS-MS analysis was performed on peptides **3c** (Figure S1) and **4f** (Figure S2). In both cases, a complete set of b-type ions were observed together with additional a-type and y-type ions (Table S1). That data allowed the sequence of the peptides and the position of the prenyl cysteine to be unambiguously established.

Assessment of a-Factor Precursor Peptides Containing Modified Isoprenoids as Substrates for Processing Enzymes. The peptide analogues described above were then evaluated as substrates for the different processing enzymes. The analogs **3b–3f** that contain a free C-terminal Cys were first tested for their ability to be methylated by Ste14. In this assay, a radiolabeled methyl group from ^{14}C-labeled SAM is transferred

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to the peptide substrate. Upon saponification, ^{14}C-labeled methanol is liberated and the resulting radioactivity is quantified via liquid scintillation counting. Analysis of reactions containing peptides **3b**–**3f** (25 μM) revealed that all of the peptides were substrates for Ste14 to varying degrees (Figure 4). Some modest differences in specific activities as compared

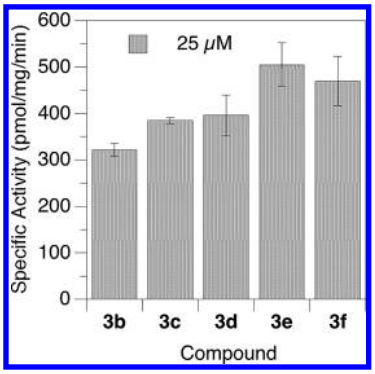

Figure 4. Assessment of **a**-factor precursor peptides **3b**–**3f** as substrates for Ste14. These assays were performed using the **a**-factor precursor peptides at a final concentration of 25 μM.

to the farnesylated **a**-factor substrate were observed. The values obtained with the alkyne-containing peptides (**3c** and **3d**) were approximately 20% higher, and those for the azide-containing peptides (**3e** and **3f**) were approximately 50% higher compared with the parent farnesylated peptide (**3b**) (Figure 4). Overall, it is clear that the peptides incorporating the non-natural isoprenoids studied here are efficiently methylated comparably to the peptide containing a natural farnesyl group (**3b**). Moreover, these results suggest that the presence of these modified isoprenoid substructures will not interfere with the methylation of larger protein substrates.

Next, endoproteolytic processing of the **a**-factor precursor peptides containing azide- and alkyne-modified isoprenoids was studied. The efficient methylation of **3b**–**3f** by Ste14 described above made it possible to employ an indirect coupled protease/methylation assay to measure Rce1 or Ste24 activity.[41,42] In this assay, if peptides **4b**–**4f** are substrates for the protease, they would be converted to the corresponding products, **3b**–**3f**. Following this cleavage, in the presence of excess Ste14 and [^{14}C]-SAM, **3b**–**3f** would be rapidly converted to **2b**–**2f** whose production could be determined via the radiolabeling methylation assay described above. Using this assay, the cleavage of peptides **4b**–**4f** by Ste24 was investigated first (Figure 5A). Analysis of reactions containing a final peptide concentration of 15 μM showed that all peptides were effectively processed by Ste24, although some relatively small differences in the specific activities were observed. Reactions performed using 5 μM peptide gave similar results, although the specific activities were approximately 2-fold lower overall. In sum, the reaction rates for Ste24 cleavage of peptides **4b**–**4f** varied less than 2-fold.

The same assay was used to explore the cleavage of peptides **4b**–**4f** by Rce1 (Figure 5B). In this case, a slightly different pattern of reactivity was observed. Using 15 μM peptide, a similar specific activity was observed for the C10Alk-functionalized peptide (**4c**) compared to the farnesylated analogue (**4b**) whereas the activity of the C15Alk-modified molecule (**4d**) was 2-fold higher. The specific activities obtained using both azide-containing peptides (**4e** and **4f**) were approximately 1.5-fold

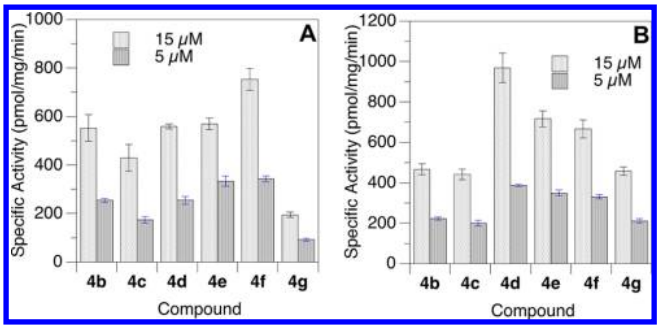

Figure 5. Assessment of **a**-factor precursor peptides **4b**–**4g** as substrates for the proteases Ste24 and Rce1. Panel A: Assays for Ste24 activity. Panel B: Assays for Rce1 activity. For each enzyme, assays were performed using either 5 μM or 15 μM peptide substrates.

higher than the farnesylated peptide **4b**. A similar pattern was observed in reactions performed using 5 μM peptide. Overall, these results with Rce1 show that peptides prenylated with alkyne- and azide-functionalized isoprenoids are processed comparably or modestly better than the peptide bearing a naturally occurring farnesyl group.

Proteolysis of a Geranylgeranylated a-Factor Precursor. While **a**-factor is normally farnesylated, many proteins are geranylgeranylated. Some of those latter proteins undergo proteolysis and methylation to yield polypeptides containing a C-terminal geranylgeranyl cysteine methyl ester, similar to the C-terminal farnesyl cysteine methyl ester present in **a**-factor. Previous work has shown that the diphosphate form of the C15Alk analog (**1d**) is a substrate for both farnesyltransferase as well as type I geranylgeranyl transferase.[26] In metabolic labeling experiments, the C15Alk group is frequently incorporated into geranylgeranylated proteins.[17] Hence, we were interested in comparing the processing of peptides bearing a geranylgeranyl group to those containing the C15Alk modification.

Since the methylation of geranylgeranylated substrates by Ste14 is well established,[43] the same coupled assay described above could be employed to study the proteolyic reactions. Accordingly, a geranylgeranylated form of an **a**-factor precursor peptide (**4g**) was prepared using the same methods outlined above for **4b**–**4f**. Using the coupled assay, we first studied the cleavage of **4g** by Ste24 to yield **3g**. Interestingly, in reactions containing 15 μM peptide, **4g** demonstrated a specific activity that was only 35% of that of the farnesylated peptide **4b** (Figure 5A); similar results were obtained using 5 μM peptide. Different results were observed with Rce1 (Figure 5B). Using 15 μM peptide, **4g** was recognized and cleaved similarly to **4b**; the same relative activity was observed using 5 μM peptide. Since the processing of the farnesylated peptide (**4b**) and those incorporating modified isoprenoids (**4c**–**4f**) was always equal to or higher than that of the geranylgeranylated peptide (**4g**), it is likely that the presence of modified isoprenoid groups in proteins that are typically geranylgeranylated would not impede their proteolysis and methylation.

Note on the Stability of Prenylated Peptides. In the course of this project and related studies, we have prepared numerous prenylated peptides containing both natural and non-natural isoprenoids. Such peptides contain thioether bonds that can be prone to sulfoxide formation as has been observed in methionine-containing peptides.[44] Prenylated peptides are generally stable to long-term storage in DMSO at −80 °C. However, in the case of peptides **4d** and **4g**, storage under

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those conditions led to some oxidation (presumably oxidation of the thioether to the corresponding sulfoxide); such oxidation upon storage in DMSO has been previously reported for related compounds. In contrast, storage in DMF appears to eliminate this problem. Consequently, we recommend that that the use of DMSO as a solvent for the preparation of solutions for long-term compound storage be avoided when working with these types of compounds.

CONCLUSIONS

In the work reported here, we first demonstrated that **a**-factor analogues that incorporate azide- and alkyne-functionalized isoprenoids manifest equal or better activity relative to the wild-type pheromone using a cell-based growth arrest assay. Next, *in vitro* experiments with partially purified enzymes indicated that peptides incorporating azide- and alkyne-functionalized isoprenoids are efficient substrates for the proteases (Rce1 and Ste24) and the methyltransferase (Ste14) responsible for the processing of prenylated peptides to their mature products. Taken together, these results suggest that metabolic labeling experiments with azide- and alkyne-functionalized isoprenoids should yield prenylated products that are fully processed and biologically functional. From a global perspective, these observations suggest that the isoprenoids studied here that incorporate bio-orthogonal functionality can be used in metabolic labeling experiments without concern that they will induce undesired physiological changes that may complicate data interpretation. It has been hypothesized that changes in the levels of prenylated proteins may be important in a variety of diseases including Parkinson's disease,[45] Alzheimer's disease,[15] neurodegeneration,[46] and viral infections.[22] Metabolic labeling with the analogues studied here could be extremely useful for detecting changes in prenylation in those diseases. Coupling metabolic labeling with quantitative mass spectrometric methods[47] is a powerful approach for measuring such changes. Efforts to implement this are in progress.

EXPERIMENTAL PROCEDURES

Synthesis. The bromides shown in Figure 2 were either purchased (**1b** and **1g**) or prepared as previously described (**1c**, **1d**, **1e**, and **1f**).[25,26,48,49] Peptide **2a** was prepared via a side-chain anchoring strategy employing Fmoc-Cys-OMe linked to trityl-resin via its side chain thiol as previously described.[34] Peptides **3a** and **4a** were prepared using standard solid-phase synthetic methods employing Wang resin. Alkylation of **2a**, **3a**, and **4a** to yield the corresponding prenylated peptides was carried out by reaction with bromides **1b**−**1g** under acidic conditions[36] to yield the corresponding peptides **2b**−**2f**, **3b**−**3g**, and **4b**−**4g**. Complete experimental details are provided in the Supporting Information.

Growth Arrest Assay. RC757 cells were cultured in YEPD (1% yeast extract, 2% peptone, 2% dextrose) and LM102 cells were cultured in MLT medium to ensure plasmid maintenance.[34] Cells were grown overnight at 30 °C with shaking in liquid medium. For use in the growth arrest assay, cells were harvested by centrifugation (1000 × g), washed twice with sterile H_2O, and resuspended to a final concentration of 1 × 10^6 cells/mL in H_2O. The cell suspension (1 mL) was combined with 3 mL of Noble agar (1.1% in water) and overlaid onto solid medium (YEPD or MLT containing 2% agar). The peptides were dissolved in CH_3OH (10 ng/μL) and diluted in 0.5% bovine serum albumin (BSA) to a final concentration of 6.4 ng/μL, and then serially diluted in 0.5% BSA to generate solutions of the desired concentrations. 2.5 μL of each dilution was spotted onto the overlay containing RC757 or LM102 cells. The plates were spotted in triplicate, and incubated 17 h at 30 °C. The experiment was repeated in quadruplicate with similar results. The end point of the assay was determined to be the lowest concentration at which a clear zone of inhibition, which indicates growth arrest, was observed.

***In Vitro* Enzymatic Activity Assays.** The assays were performed using crude membranes as previously described[41,43,50−53] with minor modifications. In brief, for the coupled proteolysis/methylation assay, the reaction contained 5 μg of Ste24 or Rce1 membranes, 10 μg of excess Ste14 membranes, 5 or 15 μM of peptide **4b**−**4g** or DMSO, 20 μM of *S*-adenosyl [^{14}C-methyl]-L-methionine (55 mCi/mmol) (PerkinElmer, USA), and 100 mM Tris-HCl, pH 7.5 in a final volume of 60 μL. For the methyltransferase assay, the reaction contained 5 μg of Ste14 membranes, 25 μM of peptide **3b**−**3f** or DMSO, 20 μM of *S*-adenosyl [^{14}C-methyl]-L-methionine in 100 mM Tris-HCl, pH 7.5, in a final volume of 60 μL. The reaction mixtures were incubated in a 30 °C water bath for 30 min and terminated with the addition of 50 μL of 1 M NaOH/1% SDS. The reaction mixtures were then spotted onto pleated filter papers. Each filter paper was lodged into the neck of a vial containing 10 mL of scintillation fluid, capped, and allowed to diffuse at room temperature for 3 h. The base-released [^{14}C]-methanol was quantified by liquid scintillation counting. Background counts from the DMSO control were subtracted from each sample. Each reaction was done in duplicate and counted three times each. The assays were repeated for a total of three trials.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00648.

Additional tables, figures, and synthetic procedures and characterization data for compounds **2a**−**2f**, **3a**−**3g**, and **4a**−**4g** (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: diste001@umn.edu. Tel (612) 624-0544.

ORCID

Christine A. Hrycyna: 0000-0001-9881-2063

Mark D. Distefano: 0000-0002-2872-0259

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

C10Alk-Br (**1c**), (2*E*,6*E*)-8-bromo-2,6-dimethyl-1-(prop-2-yn-1-yloxy)octa-2,6-diene; C15Alk-Br (**1d**), (2*E*,6*E*,10*E*)-12-bromo-2,6,10-trimethyl-1-(prop-2-yn-1-yloxy)dodeca-2,6,10-triene; C15Az-Br (**1e**), (2*E*,6*E*,10*E*)-1-azido-12-bromo-2,6,10-trimethyldodeca-2,6,10-triene; C15dhAz-Br (**1f**), (2*E*,6*E*)-12-azido-1-bromo-3,7,11-trimethyldodeca-2,6-diene; ESI-MS, Electrospray ionization mass spectrometry; Fmoc, Fluorenylmethyloxycarbonyl; FPP, Farnesyl diphosphate; GGPP, Geranylgeranyl diphosphate; HCTU, *O*-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; MS, Mass spectrometry; RP-HPLC, Reversed-phase high performance liquid chromatography; SAM, *S*-Adenosyl methionine; SPPS, Solid-phase peptide synthesis; Trt, Triphenylmethyl

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