Diazirine-Containing Photoactivatable Isoprenoid: Synthesis and Application in Studies with Isoprenylcysteine Carboxyl Methyltransferase

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Supporting Information

**ABSTRACT:** Photoaffinity labeling is a useful technique employed to identify protein–ligand and protein–protein noncovalent interactions. Photolabeling experiments have been particularly informative for probing membrane-bound proteins where structural information is difficult to obtain. The most widely used classes of photoactive functionalities include aryl azides, diazocarbonyls, diazirines, and benzophenones. Diazirines are intrinsically smaller than benzophenones and generate carbenes upon photolysis that react with a broader range of amino acid side chains compared with the benzophenone-derived diradical; this makes diazirines potentially more general photoaffinity-labeling agents. In this article, we describe the development and application of a new isoprenoid analogue containing a diazirine moiety that was prepared in six steps and incorporated into an α-factor-derived peptide produced via solid-phase synthesis. In addition to the diazirine moiety, fluorescein and biotin groups were also incorporated into the peptide to aid in the detection and enrichment of photo-cross-linked products. This multifunctional diazirine-containing peptide was a substrate for Ste14p, the yeast homologue of the potential anticancer target Icmt, with K_m (6.6 μM) and V_max (947 pmol min⁻¹ mg⁻¹) values comparable or better than α-factor peptides functionalized with benzophenone-based isoprenoids. Photo-cross-linking experiments demonstrated that the diazirine probe photo-cross-linked to Ste14p with observably higher efficiency than benzophenone-containing α-factor peptides.

**INTRODUCTION**

Photoaffinity labeling is a useful technique employed to identify noncovalent interactions between molecules, including protein–ligand and protein–protein complexes. The utility of photoreactive probes to study such interactions is highly dependent upon three criteria, including how closely the photolabeling moiety mimics the native structure, what type of reactive intermediates are formed, and the spatial relationship between the two interacting molecules. The most widely used classes of photoactive functionality include aryl azides, diazocarbonyls and diazirines, and benzophenones, which give rise to nitrenes, carbenes, and diradicals, respectively, upon photolysis.¹

Prenylated proteins are a class of membrane-associated polyepitides that employ a farnesyl or geranylgeranyl isoprenoid (sometimes two in the latter case) to anchor the proteins into membranes. A three-step process consisting of prenylation, proteolysis, and carboxymethylation is frequently required to produce the final mature active proteins. Prenylation is performed by farnesyl- or geranylgeranyltransferases, and proteolysis is carried out by Rce1 or Ste24, whereas carboxymethylation is catalyzed by Icmt, a SAM-dependent enzyme.² Attachment of the hydrophobic isoprenoid causes membrane association³ and is essential for membrane-associated functions involving protein–protein interactions,⁴ signal transduction,⁵,⁶ and cellular homeostasis regulation.⁷ Because of the fact that Ras prenylation is required for its proper cellular localization and function,⁸ numerous human clinical trials have been conducted to inhibit this critical signal transduction event that is particularly relevant to cancer.⁹,¹⁰ Importantly, an area of growing interest is developing inhibitors for the other enzymes in the processing pathway including Lcmt, which is localized to the membrane of the endoplasmic reticulum.

Photoactive analogues of farnesyl diphosphate have been used extensively to study the interactions between soluble proteins and their ligands, including isoprenoids and prenyltransferases. This class of photoprobes includes molecules containing aryl azides,¹¹ diazoesters,¹²−¹⁵ and benzophenones.
nones,16−20 with the latter being the most commonly employed. Peptides containing these photoactive isoprenoids have also been used to study the interactions between prenylated proteins and their cognate receptors.21 Recently, we used peptides functionalized with benzophenone-containing isoprenoids in photo-cross-linking experiments with membrane-associated proteins that also recognize isoprene moieties, including the Ras-converting enzyme Rce1 and the isoprenylcysteine carboxyl methyltransferase, Icmt, two enzymes involved in the CaaX protein post-translational processing pathway.22,23 Although we showed that those enzymes could be successfully photolabeled, the yield of cross-linking was not sufficient to generate quantities of material adequate for mass spectrometric sequencing. In addition, the benzophenone-containing peptides were generally poorer substrates than those incorporating a natural farnesyl group, likely because of the larger size of the benzophenone moiety. To address this size question and potentially increase the yield of cross-linked protein, we wanted to explore the use of smaller isoprene unit surrogates. Although a number of diazoester-containing isoprenoid analogues have been previously reported,12,13,17,19 their synthesis is complicated by the need to use phosgene gas and trifluorodiazoethane.24 In contrast, diazirines are much simpler to prepare from ketones. Because diazirines are intrinsically smaller than benzophenones and more closely approximate the size and shape of an isoprene unit, they should be effective isoprenoid mimics; moreover, because they generate carbenes upon photolysis, they also have the potential to react with a wider range of amino acid side chains, thereby increasing photo-cross-linking efficiency.

Herein, we describe the synthesis of a diazirine-containing isoprenoid unit (1) (Figure 1) and its incorporation into a biotinylated and fluorescently labeled α-factor peptide analogue (16) (Figure 2); the dodecapeptide, α-factor, is a naturally occurring farnesylated peptide found in yeast and known to be generated via methylation by Icmt.25 The ability of this multifunctional photactive peptide to bind and serve as a substrate for Icmt was then evaluated using Ste14p, the Icmt from the yeast Saccharomyces cerevisiae, as a model enzyme. The diazirine-containing α-factor analogue demonstrated an increased V_{max} and lower K_{m} relative to benzophenone-containing probes. Cross-linking studies showed that the efficiency of photolabeling achieved with the diazirine-based probe was greater than that of benzophenone-containing probes. In contrast to previous work where antibody-based methods were necessary for detection of cross-linked protein, in-gel fluorescence via a fluorophore incorporated into the peptide was used here to detect the cross-linked purified enzyme successfully. These results suggest that such multifunctional diazirine-containing peptides should be useful for identifying active-site residues in Icmts and potentially other enzymes involved in the processing of prenylated proteins.

**RESULTS AND DISCUSSION**

**Design and Synthesis of Diazirine-Containing Isoprenoid.** To prepare the desired multifunctional α-factor peptide for in vitro kinetic and photo-cross-linking analyses of Ste14p, the initial strategy was first to design an isoprenoid moiety containing a diazirine group. Compound 1 was selected as the target based on the overall similarity in size of the analogue compared with farnesyl diphosphate (FPP). Comparison of 1 with benzophenone- and DATFP-containing FPP analogues (2 and 3) reveals that 1 is closest in size to FPP (Figure 1). Although potentially less stable than ether-linked analogues such as 2, the ester analogue 1 was chosen for ease of synthesis. A convergent strategy was used to prepare 1 based on previously published routes for the production of the

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**Figure 1.** Photoactivatable isoprenoid analogues. Left: farnesol, diazirine 1, benzophenone 2, and DATFP 3. Right: space-filling models of the isoprenoid analogues. Hydrogen atoms are omitted for clarity. Color scheme: carbon, green; oxygen, red; nitrogen, blue; and fluorine, white. Spheres are shown at 0.6 van der Waals radii for clarity.

**Figure 2.** α-Factor-based peptides for the in vitro study of Icmt.
isoprenoid building block, 9,17 and the diazirine-derivative pentanoic acid, 6 (Scheme 1).26 Coupling of 9 and 6 in the presence of DIC and DMAP afforded the ester (10). Removal of the THP protecting group was performed with PPTS in EtOH followed by conversion of the free alcohol, 11, to the corresponding bromide, 12, by reaction with CBr4 and PPh3. Purification of the bromide was carried out using a small reversed-phase cartridge to minimize decomposition of the allylic bromide on silica gel. The reversed-phase purification also appears to remove colored impurities that interfere with the subsequent peptide alkylation; although this process does not remove unreacted alcohol starting material, that compound does not interfere with the subsequent alkylation chemistry.

**Synthesis of Prenylated a-Factor.** We incorporated isoprenoid analogue 12 into a peptide based on the structure of the farnesylated dodecapeptide mating pheromone a-factor, an in vivo substrate for Ste14p that is produced via the same three-step process as larger prenylated proteins (Scheme 2).25 This peptide (16) contains a free C-terminal carboxylate to serve as the methyl acceptor in the enzymatic reaction. Solid-phase peptide synthesis was used to construct peptide utilizing standard Fmoc/HCTU coupling conditions. An additional N-terminal lysine residue was added to the a-factor sequence to provide a second handle (via the side chain) for fluorophore attachment. After the peptide was elongated, its free N-terminus was biotinylated to yield 14. Biotin-Peg4-NHS was chosen for installation of the biotin label because of its efficient reaction kinetics and inexpensive cost. After specific on-resin cleavage of the Dde protecting group from the side-chain α-amino group of the N-terminal lysine with a 10% hydrazine/DMF solution, the resulting free amine was acylated with 5-carboxyfluorescein succinimidyl ester (5-Fam) in the presence of DIEA to give the fluorescently labeled peptide on resin. 5-Fam was chosen as the fluorescent reporter because of its reactivity with primary amines via the succinimidyl ester and its convenient emission wavelength that makes it easy to detect using most commercially available fluorescence scanners. Cleavage from the resin and acidic deprotection was carried out simultaneously by treatment with Reagent K to afford an orange peptide 15, with a C-terminal cysteine bearing a free thiol. It is interesting to note that previous reports have noted that some epimerization of C-terminal cysteine residues can occur when Cys-functionalized Wang resin and Reagent K cleavage conditions are used,27 however, after RP-HPLC purification, no evidence for epimerization was observed. MS/MS analysis (see Supporting Information Figure S5) revealed a large ensemble of b-type and y-type fragments consistent with the proposed structure for 15. Alkylation of the free thiol was performed using bromide 12 (1.5 equiv) in the presence of Zn(OAc)2 (0.1 equiv) in acidic DMF to yield prenylated peptide 16, whose identity was determined via ESI-MS and purity was confirmed by analytical RP-HPLC. As was observed for 15, MS/MS analysis of 16 (see Supporting Information Figure S6) showed the presence of a variety of b-type and y-type fragments, consistent with the proposed structure for 16. Fragmentation via loss of the farnesyl group was also observed as had been previously reported for prenylated peptides.23,28 Farnesylated control peptide 17 and benzophenone-containing peptide 19 were prepared by a similar procedure, whereas 18 was prepared as previously described.22
Table 1. In Vitro Reaction Kinetics for a-Factor Peptides Methylated by His-Ste14p

| compound | \( V_{max} \) (pmol min\(^{-1}\) mg\(^{-1}\))\(^a\) | \( K_{m(app)} \) (μM)\(^b\) | \( V_{max}/K_{m} \) (pmol min\(^{-1}\) mg\(^{-1}\) μM\(^{-1}\))\(^a\) | \( K_{m} \) (μM) \( V_{max}/K_{m} \) (pmol min\(^{-1}\) mg\(^{-1}\) μM\(^{-1}\))\(^a\) |
|----------|-----------------|-----------------|-----------------|-----------------|
| AFC\(^{22}\) | 870 ± 15 | 15.9 ± 0.9 | 54 | |
| 16 | 947 ± 4 | 6.6 ± 0.2 | 143 | |
| 17 | 1919 ± 30 | 12.2 ± 0.1 | 157 | |
| 18\(^{22}\) | 762 ± 28 | 14.6 ± 0.3 | 52 | |
| 19 | 217 ± 3 | 27.8 ± 1 | 7.0 | |

\(^{a}\)Reported as pmol of methyl groups transferred to substrate. \(^b\)Because these experiments were performed at a single concentration of SAM and the biphasic nature of the membrane suspension makes the concentration of the peptide substrate unknown (because of partitioning into the lipid membranes), these experiments provide only apparent values for \( K_{m} \).
each probe that was ~2–8 times greater than their individual $K_m$ values, the increase in labeling is likely not attributable to differences in affinities between the different probes. Rather, it must be due to either the increased reactivity of the carbene intermediate generated from diazirine 16 compared to the diradical intermediate produced from the benzophenone photophores of 18 and 19 or the greater proximity or more favorable geometry of the peptide in the active site. Currently, additional diazirine- and benzophenone-containing probes are being prepared, and we will determine whether the former types always yield higher levels of cross-linking.

Lastly, in addition to confirming the formation of the cross-linked protein, the specificity of the photolabeling of His-Ste14p with 16 was examined in a competition experiment (Figure 4). A biotinylated a-factor precursor peptide, 17, was chosen as a competitor, as it was a substrate for His-Ste14p (Table 1) and closely mimicked the structure of a-Ste14 antibody. The data shown is from one of two replicates of this experiment.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| Ste14p | + | + | + | + | + | + | + | + |
| 16 (μM) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 17 (μM) | - | - | 5 | 10 | 25 | 50 | 100 | 200 |
| UV light | + | + | + | + | + | + | + | + |

Figure 4. Competition of photolabeling of His-Ste14p by 16 using a biotinylated a-factor precursor peptide 17. For this experiment, His-Ste14p crude membrane protein (100 μg) was mixed with 16 and 17 at the concentrations indicated and irradiated on ice for 30 min followed by enrichment with neutravidin-agarose beads. The samples were then eluted and resolved via SDS-PAGE. The resulting gel was blotted to a nitrocellulose membrane and visualized using an α-Ste14 antibody. The data shown is from one of two replicates of this experiment.

**CONCLUSIONS**

Herein, we have described the development and application of a new class of isoprenoid analogue containing a photoexcitable diazirine moiety. The photoactive farnesyl mimick was prepared in six steps and incorporated into a multifunctional peptide produced via solid-phase synthesis. Kinetic analyses with His-Ste14p showed that the diazirine-containing peptide was an efficient substrate for the enzyme compared to similar peptides functionalized with benzophenone-based isoprenoids. The diazirine-based probe cross-linked to His-Ste14p upon UV irradiation with an observable increase in efficiency compared to benzophenone-containing peptides. Lastly, the incorporation and use of this diazirine-modified isoprenoid in a peptide probe equipped with a fluorophore greatly simplified the detection of cross-linked products. The greater yield of photo-cross-linked His-Ste14p coupled with the ease of analysis obtained with this new class of isoprenoid mimics should facilitate the identification of active-site residues in His-Ste14p. Such experiments are currently underway for this important membrane protein target. Given their improved features compared with other types of photoactive isoprenoid probes, this new class of analogues should be useful for a variety of studies of enzymes that act on terpene-derived molecules.

### EXPERIMENTAL SECTION

**General Information.** All solvents and reagents used for the synthesis of the diazirine isoprenoid analogue and solid-phase peptide synthesis of the photoactivatable peptides were of analytical grade and purchased from Peptides International (Louisville, KY), NovaBioChem (Nohenbrunn, Germany), or Sigma-Aldrich (St. Louis, MO). NHS-PEG2-Biotin was obtained from Thermo Scientific. The benzophenone-containing isoprenoid bromides, C6p-m-Bp-Br (2), was prepared as previously described.13−27 HR-ESI-MS analysis was performed using a Bio-TOF-II mass spectrometer.

3-(3-Methylaziridin-3-yl)propanoic Acid (5). Leucinal acid 4 (1.61 g, 13.8 mmol, 1 equiv) was dissolved in 7 N NH3 in CH3OH (13.5 mL, 91.0 mmol, 7 equiv). The resulting solution was stirred under N2 on ice for 3 h. A solution of hydroxylamine-O-sulfonic acid (1.80 g, 15.9 mmol, 1.2 equiv) in CH3OH (12 mL) was added dropwise at a rate of 1 s−1. The reaction mixture was stirred for 20 h and allowed to warm to rt. N2 was bubbled through the solution for 1 h to remove NH3 gas. Vacuum filtration and concentration resulted in a yellow oil that was used in the next step without purification.

3-(3-Methyl-3H-diazirin-3-yl)propanoic Acid (6). Diazirine 5 was dissolved in CH3OH (10 mL) and stirred on ice for 5 min in a tin foil-covered flask. Triethylamine (3.00 mL, 21.5 mmol) was added and allowed to stir for 5 min. Slowly, chips of I2 were added until the solution remained a brown-red color for longer than 5 min after the last addition. The reaction solution was diluted with EtOAc and washed with 1 M HCl and aqueous 10% sodium thiosulfate until the organic layer was colorless. The aqueous layer was further extracted with EtOAc and concentrated to dryness. The residue that was used in the next step without purification.

(E)-2-(3,7-Dimethylocta-2,6-dien-1-yl)oxytetrahydro-2H-pyran (8). This compound was prepared via a modification of a previously described procedure.28 To a solution of geraniol 7 (3.11 g, 20.2 mmol, 1 equiv) in CH2Cl2 (3.5 mL) were added DHP (2.54 g, 30.3 mmol, 1.5 equiv) and PPTS (0.502 g, 2.0 mmol, 0.1 equiv), and the resulting solution was stirred at room temperature. The reaction mixture was quenched with saturated aqueous NaHCO3 and extracted with CH2Cl2. The organic layer was dried over MgSO4 and concentrated to yield a yellow oil that was purified via silica gel chromatography (EtOAc:Hexane = 1:1). The resulting product was obtained in 97% yield.

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and dried over MgSO₄. After concentration, the residue was purified by flash chromatography (hexanes/EtOAc, 3:2, v/v) on silica gel to obtain 2.36 g (46%) of alcohol 9 as a clear oil. ¹H NMR (300 MHz, CDCl₃): δ 1.65 (s, 6H), 1.48–1.85 (m, 6H), 2.04–2.22 (m, 4H), 3.47–3.54 (m, 1H), 3.85–3.92 (m, 1H), 3.91 (s, 2H), 3.98–4.05 (dd, 1H, J = 7.2, 12 Hz), 4.21–4.27 (dd, 1H, J = 6.3, 12 Hz), 4.62 (t, 1H, J = 2.7 Hz), 5.36 (t, 1H, J = 6.3 Hz). HR-ESI-MS: calcd for C₈H₁₀NO₂Na [M + Na]+ 287.1853; found, 287.1790.

(2E,6E)-2,6-Dimethyl-8-(2-hydroxyocta-2,6-dien-1-yl)octa-2,6-dien-1-yl 3-(3-Methyl-3H-diazirin-3-yl)propanoate (11). To a solution of 10 (99 mg, 0.271 mmol, 1 equiv) in EtOH (1.60 mL) was added PPTS (99 mg, 0.271 mmol, 1 equiv) in EtOH (1.60 mL). The resulting solution was allowed to stir for 2 h at rt. After the reaction mixture was filtered and concentrated. Purification by flash chromatography (hexanes/EtOAc, 5:1) afforded 1.07 g (57%) of diazirine 6 as a clear oil. ¹H NMR (300 MHz, CDCl₃): δ 1.03 (s, 3H), 1.48–1.85 (m, 14H), 2.04–2.22 (m, 4H), 3.47–3.54 (m, 1H), 3.85–3.92 (m, 1H), 3.98–4.05 (dd, 1H, J = 7.2, 12 Hz), 4.21–4.27 (dd, 1H, J = 6.2 Hz), 5.36 (t, 1H, J = 6.3 Hz). ¹C NMR (75.0 MHz, CDCl₃): δ 13.6, 16.4, 23.5, 26.4, 32.9, 39.7, 44.2, 58.9, 69.3, 72.1, 124.6, 130.4, 130.7, 141.8, 173.1. IR (NaCl, cm⁻¹): 2925 (m), 2870 (m), 1736 (s), 1656 (w), 1586 (w), 1446 (m), 1317.1223; found, 1317.1172.

Synthesis of Biotin-Peg₄-K(5-Fam)YIIKGVFWDPAC-(Fr)-OH (17). Peptide synthesis was carried out using an automated solid-phase peptide synthesizer (PS3, Protein Technologies Inc., Memphis, TN) employing standard Fmoc/HCTU-based chemistry. Synthesis began with the free thiol-containing peptide (20 mg, 13 μmol, 1 equiv) catalyzed by DIEA (14.4 μL, 83 μmol, 1 equiv) for 16 h. After Reagent K cleavage and HPLC purification, the thiol-containing peptide was further purified by preparative reverse-phase HPLC on a C18 column using a gradient of 0%–30% solvent B over 20 min at a flow rate of 3 mL/min. Fractions were analyzed using an analytical C18 RP-HPLC column employing a linear gradient (0–100% solvent B over 60 min at a flow rate of 1 mL/min) and detected at 214 nm. Fractions containing peptide product of at least 90% purity were pooled, lyophilized, and re-dissolved in DMF (0.50 mL) and loaded onto a C18 Sep-Pak column with 3.0 mL of DMF directly into the reaction mixture. The reaction was analyzed by analytical RP-HPLC, purified via ESI-TOF MS. This peptide was then reacted with 5-FAM SE (45 mg, 86.0 μmol, 1 equiv) using the same conditions as above (5% hydrazine in DMF, 20 min) except reaction was divided into three portions for further synthesis on a reduced scale. Using 83.0 μmol of peptide, the free amino terminus was biotinylated in DMF (5 mL) with NHS-PEG₂-Biotin (0.49 mg, 83.0 μmol, 1 equiv) catalyzed by DIEA (14.4 μL, 83 μmol, 1 equiv) for 16 h. After acylation, the resin-bound peptide was washed thoroughly with CH₂Cl₂ and dried in vacuo for 4 h. The peptide was then reacted with 5% hydrazine in DMF (5 mL, v/v) to orthogonally remove the protected side chain. After verifying the deprotection was complete by ninhydrin analysis, the peptide was washed with CH₂Cl₂, dried, and then reacted 5-FAM SE (45 mg, 86.0 μmol, 1 equiv) catalyzed by DIEA (14.4 μL, 83 μmol, 1 equiv) overnight. The peptide was cleaved from the resin along with simultaneous side-chain deprotection by treatment with Reagent K containing TFA (10 mL), crystalline phenol (0.5 g), 1,2-ethanediol (0.25 mL), thioanisole (0.5 mL), and H₂O (0.5 mL) for 2 h at rt. The released peptide was collected and combined with TFA washes of the resin before precipitation of the peptide in chilled Et₂O (100 mL). The crude solid peptide was collected by centrifugation, the supernatant was removed, and the resulting pellet was washed twice with cold Et₂O (50 mL), repeating the centrifugation and supernatant-removal steps each time. The crude peptide was purified using a semipreparative C₁₈ RP-HPLC column with detection at 280 nm and eluted with a gradient of solvent A (H₂O/0.1% TFA, v/v) and solvent B (CH₂CN/0.1% TFA, v/v). The peptide (150 mg) was dissolved in a DMF/H₂O solution (1.5 v/v, 25 mL) and applied to the column equilibrated in solvent A, and washed with 15% solvent B for 15 min. The peptide was eluted using a linear gradient of (15–65% solvent B over 1.5 h at a flow rate of 5 mL/min). Fractions were analyzed using an analytical C₁₈ RP-HPLC column employing a linear gradient (0–100% solvent B in 60 min at a flow rate of 1 mL/min) and detected at 214 nm. Fractions containing peptide product of at least 90% purity were pooled, lyophilized, and re-dissolved in 0.50 mL of DMF and loaded onto a C₁₈ Sep-Pak column that had been equilibrated with 5% CH₂CN in aqueous 10% TFA. The solution was washed with 5% CH₂CN (5 mL) followed by 30% CH₂CN (10 mL). The purified peptide was then eluted from the column with 3.0 mL of DMF directly into the reaction flask that contained the dissolved peptide. Zn(OAc)₂·2H₂O (5.4 mg, 25 μmol, 5 equiv) was then added to initiate the alkylation reaction. After 4 h, the reaction was analyzed by analytical RP-HPLC, purified by semi-preparative C₁₈ RP-HPLC, and identified via ESI-TOF MS. This reaction yielded 5.1 mg (21%) of desired alkylated peptide 16. Purity by HPLC: 96.1%. ESI-MS: calcd for C₁₅₃H₂₀₇N₄₀O₃₂S₂ [M + 2H]+ 1186.0334; found, 1186.0453.

Synthesis of Biotin-Peg₄-YIIKGVFWDPAC(10)-Diazirine)-OH (16). Peptide 15 (20 mg, 5.3 μmol, 1 equiv) was dissolved in DMF/n-butanol/H₂O (0.10% TFA) (3:1:1, v/v/v, 6 mL). Isopropenyl bromide 12 (15 mg, 54.5 μmol, 10 equiv) was dissolved in 0.50 mL of DMF and loaded onto a C₁₈ Sep-Pak column that had been equilibrated with 5% CH₂CN in aqueous 10% TFA. The column was washed with 5% CH₂CN (5 mL) followed by 30% CH₂CN (10 mL). The purified bromide was then eluted from the column with 3.0 mL of DMF directly into the reaction flask that contained the dissolved peptide. Zn(OAc)₂·2H₂O (5.4 mg, 25 μmol, 5 equiv) was then added to initiate the alkylation reaction. After 4 h, the reaction was analyzed by analytical RP-HPLC, purified by semi-preparative C₁₈ RP-HPLC, and identified via ESI-TOF MS. This reaction yielded 5.1 mg (21%) of desired alkylated peptide 16. Purity by HPLC: 96.1%. ESI-MS: calcd for C₁₅₃H₂₀₇N₄₀O₃₂S₂ [M + 2H]+ 1186.0334; found, 1186.0453.

Synthesis of Biotin-Peg₄-YIIKGVFWDPAC(15)-Diazirine)-OH (17). Solid-phase peptide synthesis and purification were carried out in the same fashion as described above. Following complete chain elongation, the peptide’s N-terminus was deprotected with 10% piperidine in DMF (v/v), and the presence of the resulting free amine was confirmed by ninhydrin analysis. Using 83.0 μmol of peptide, the free amino terminus was biotinylated in DMF (5 mL) with NHS-PEG₂-Biotin (0.49 mg, 83.0 μmol, 1 equiv) catalyzed by DIEA (14.4 μL, 83 μmol, 1 equiv) for 16 h. After Reagent K cleavage and HPLC purification, the thiol-containing peptide (20 mg, 13 μmol, 1 equiv) was prenylated with farnesyl bromide (10 mg, 65 μmol, 5 equiv) using the same conditions as above (66% yield by HPLC: 92.9%). ESI-MS: calcd for C₁₅₂H₂₁₀N₄₀O₃₂S₂ [M + 2H]+ 1045.0490; found, 1045.0491.
with replacement of the isoprenoid analogue 12 with C12-m-Bp-Bt that was prepared as previously described.22 Purity by HPLC: 94.2%. ESI-MS: calcd for C15H27N3O3S2 [M + 2H]+, 1325.0951; found, 1325.0994.

Protein Isolation and in Vitro Methyltransferase Vapor-Diffusion Assays. For experiments using crude protein, membrane preparations from the yeast strain CH2704 overexpressing His-Ste14p were prepared as previously described, with minor modifications.23 Following centrifugation at 100 000g for 1 h, the membrane pellet was resuspended in 10 mM Tris-HCl, pH 7.5, aliquoted, flash-frozen in liquid N2, and stored at -80 °C. In vitro assays for methyltransferase activity were performed using crude membranes as previously described.24 In brief, reactions contained crude membrane preparations, 200 μM AFC, 20 μM S-adenosyl-l-[methyl-14C]methionine (14C)SAM) (50–60 μCi/mmol), and 100 mM Tris-HCl, pH 7.5, in 60 μL. The reactions were incubated at 30 °C for 30 min and terminated by the addition of 50 μL of 1 M NaOH and 1% SDS (v/v). Each reaction (100 μL) was then spotted onto a filter paper that was wedged into the neck of a vial containing 10 mL of scintillation fluid and allowed to dry-wedge into the neck of a vial containing 10 mL of scintillation fluid. For experiments performed with purified His-Ste14p, His10myc3N-Ste14p (His-Ste14p) was expressed in a deletion strain30 and purified as previously described.7

Photo-Cross-Linking and Neutravidin-Agarose Pulldown Assays. Photo-cross-linking assays were performed as described previously, with minor modifications.20,22 One-hundred micrograms of crude membrane preparation expressing His-Ste14p in 10 mM Tris-HCl, pH 7.5, was preincubated with increasing concentrations of the competition peptide before addition of the photolabeling reagent and incubated at 4 °C for 5 min. The samples were irradiated at 365 nm in 96-well plates for 30 min on ice. The resulting protein samples were solubilized in 800 μL of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecylsulfate)/10% SDS and incubated with 50 μL of a 50% neutravidin/RIPA bead slurry for 2 h at 4 °C. The beads were centrifuged at 13 000g for 1 min and washed three times with RIPA/10% SDS. The cross-linked protein was eluted from the neutravidin beads by the addition of 50 μL of 2X SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 30% sucrose (w/v), 10% sodium dodecylsulfate (w/v), 3.5 M 2-mercaptoethanol, and 0.1% bromophenol blue (w/v)). Samples were heated for 30 min at 65 °C and subjected to 12% SDS-PAGE and immunoblot analysis.

Fluorescence Imaging. SDS-PAGE gels were imaged using GE Healthcare Life Sciences Typhoon Trio scanner. Excitation was performed at 488 nm, and emission was observed with a 520 nm bandpass emission filter (520 BP 40). The images were collected with a pixel size of 50 μm at normal sensitivity with a PMT voltage of 400 V.

Immunoblot Analysis. Proteins from SDS-PAGE gels were transferred to nitrocellulose membranes (0.22 μm), and the membranes were blocked at rt for 2 h in 20% (w/v) nonfat dry milk in phosphate-buffered saline with Tween-20 (137 mM NaCl, 2.7 mM KCl, 4 mM Na2HPO4, 1.8 mM KH2PO4, and 0.05% (v/v) Tween-20, pH 7.4) (PBST). The blocked membrane was incubated for 2 h at rt with an α-Ste14p (1:500-crude or 1:10 000-pure protein) antibody in 5% (w/v) nonfat dry milk in PBST. The membrane was washed in PBST three times and incubated for 1 h at rt with goat α-rabbit IgG-HRP (1:10 000) in 5% (w/v) dry milk in PBST. Incubation of the membrane with NeutrAvidin HRP (1:5000) was performed in 5% (w/v) BSA in PBST for 3 h at rt. The membranes were washed three times with PBST, and the protein bands were visualized using ECL.

ASSOCIATED CONTENT

Supporting Information

1H NMR, 13C HMR, and IR for all new compounds; ESI-MS and analytical HPLC chromatograms for reported peptides; and analysis of cross-linking reactions containing purified His-Ste14p and photoactive probes 16 and 19 after pulldown with streptavidin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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