

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

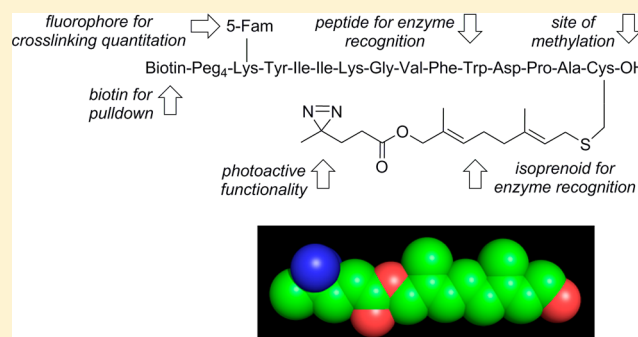
Jeffrey S. Vervacke,<sup>†</sup> Amy L. Funk,<sup>‡</sup> Yen-Chih Wang,<sup>†</sup> Mark Strom,<sup>†</sup> Christine A. Hrycyna,<sup>\*,‡</sup> and Mark D. Distefano<sup>\*,†</sup>

<sup>†</sup>Departments of Chemistry and Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, United States

<sup>‡</sup>Department of Chemistry, Purdue University, West Lafayette, Indiana 47907–2084, United States

## 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 a-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  $\mu\text{M}$ ) and  $V_{\text{max}}$  (947  $\text{pmol min}^{-1} \text{mg}^{-1}$ ) values comparable or better than a-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 a-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.<sup>1</sup>

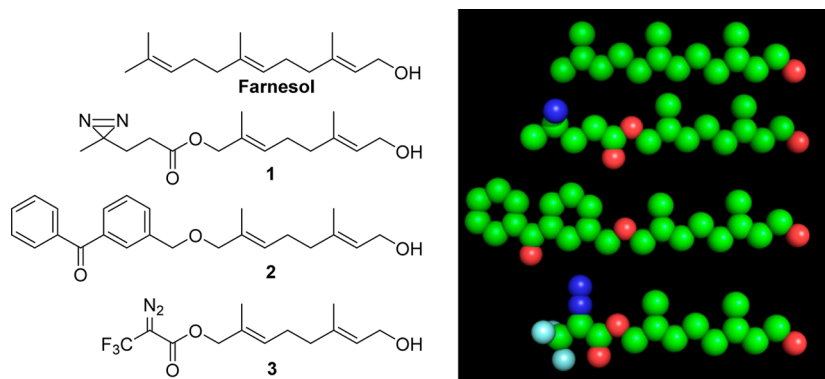
Prenylated proteins are a class of membrane-associated polypeptides that employ a farnesyl or geranylgeranyl isoprenoid (sometimes two in the latter case) to anchor the proteins into membranes. A three-step process consisting of prenylation, proteolysis, and carboxymethylation is frequently required 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.<sup>2</sup> Attachment of the hydrophobic isoprenoid causes membrane association<sup>3</sup> and is essential for membrane-associated functions involving protein–protein interactions,<sup>4</sup> signal transduction,<sup>5,6</sup> and cellular homeostasis regulation.<sup>7</sup> Because of the fact that Ras prenylation is required for its proper cellular localization and function,<sup>8</sup> numerous human clinical trials have been conducted to inhibit this critical signal transduction event that is particularly relevant to cancer.<sup>9,10</sup> Importantly, an area of growing interest is developing inhibitors for the other enzymes in the processing pathway including Icmt, 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,<sup>11</sup> diazoesters,<sup>12–15</sup> and benzophe-

Received: November 22, 2013

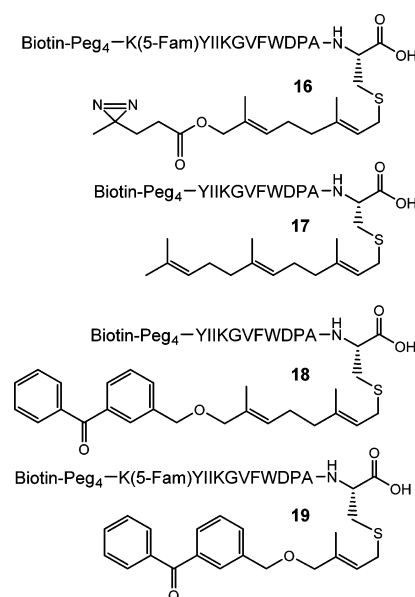
Published: February 6, 2014



**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.

nones,<sup>16–20</sup> 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.<sup>21</sup> 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.<sup>22,23</sup> 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,<sup>12,13,17,19</sup> their synthesis is complicated by the need to use phosgene gas and trifluorodiazethane.<sup>24</sup> 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  $\alpha$ -factor peptide analogue (**16**) (Figure 2); the dodecapeptide,  $\alpha$ -factor, is a naturally occurring farnesylated peptide found in yeast and known to be generated via methylation by Icmt.<sup>25</sup> The ability of this multifunctional photoactive 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  $\alpha$ -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



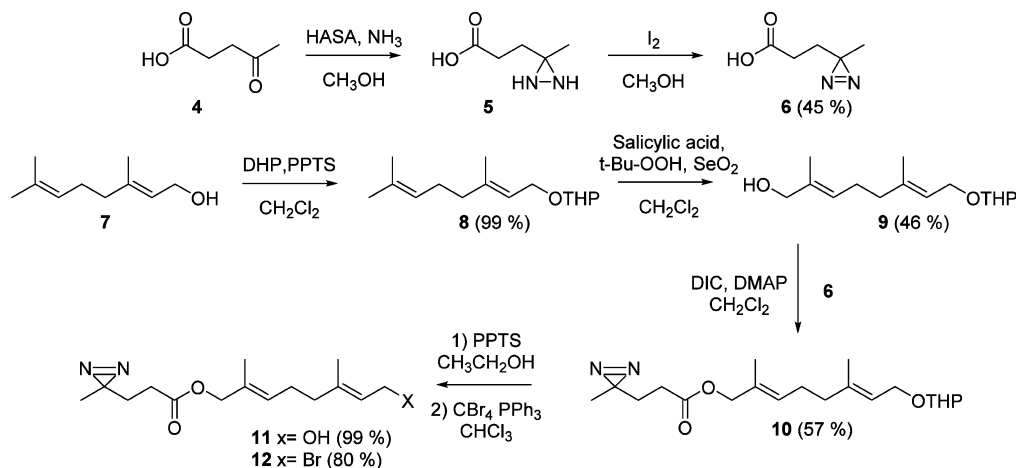
**Figure 2.**  $\alpha$ -Factor-based peptides for the in vitro study of Icmt.

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  $\alpha$ -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

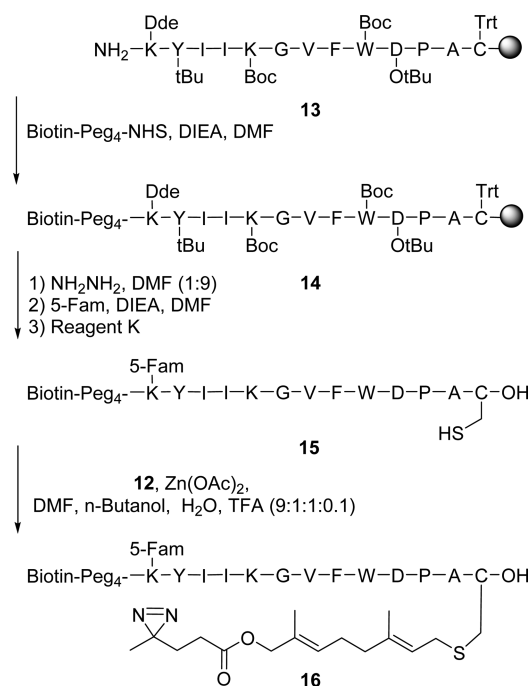
Scheme 1. Synthesis of a Diazirine-Containing Analogue of a Farnesyl Group



isoprenoid building block, **9**,<sup>17</sup> and the diazirine-derivative pentanoic acid, **6** (Scheme 1).<sup>26</sup> 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  $\text{CBr}_4$  and  $\text{PPh}_3$ . 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).<sup>25</sup> 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 **13** 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-Peg<sub>4</sub>-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  $\epsilon$ -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;<sup>27</sup> however, after RP-HPLC

Scheme 2. Synthesis of Prenylated a-Factor Precursor Peptide **16**

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  $\text{Zn}(\text{OAc})_2$  (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 has been previously reported for prenylated peptides.<sup>23,28</sup> Farnesylated control peptide **17** and benzophenone-containing peptide **19** were prepared by a similar procedure, whereas **18** was prepared as previously described.<sup>22</sup>

Table 1. In Vitro Reaction Kinetics for a-Factor Peptides Methylated by His-Ste14p

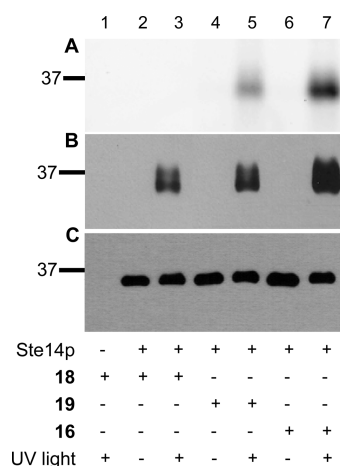
compound	$V_{\max}$ (pmol min <sup>-1</sup> mg <sup>-1</sup> ) <sup>a</sup>	$K_{m(\text{app})}$ ( $\mu\text{M}$ ) <sup>b</sup>	$V_{\max}/K_m$ (pmol min <sup>-1</sup> mg <sup>-1</sup> $\mu\text{M}^{-1}$ ) <sup>a,b</sup>
AFC <sup>22</sup>	870 $\pm$ 15	15.9 $\pm$ 0.9	54
16	947 $\pm$ 4	6.6 $\pm$ 0.2	143
17	1919 $\pm$ 30	12.2 $\pm$ 0.1	157
18 <sup>22</sup>	762 $\pm$ 28	14.6 $\pm$ 0.3	52
19	217 $\pm$ 3	27.8 $\pm$ 1	7.0

<sup>a</sup>Reported as pmol of methyl groups transferred to substrate. <sup>b</sup>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  $K_m$ .

**Diazirine-Modified a-Factor Is a Substrate for His-Ste14p.** To assess the ability of **16** to act as a substrate for Icmt, we used a recombinant form of yeast Icmt (Ste14p) that incorporates both a His<sub>10</sub> tag for purification and a triply iterated *myc* tag to facilitate detection by immunoblot analysis (His-Ste14p).<sup>7</sup> The ability of **16** to act as a substrate for His-Ste14p was determined using an in vitro methyltransferase vapor-diffusion assay.<sup>7</sup> The  $K_m$  and  $V_{\max}$  values (Table 1) for **16** were measured using crude membrane fractions prepared from yeast expressing His-Ste14p using *N*-acetyl-S-farnesyl-L-cysteine (AFC) as a positive control. Comparison of the kinetic parameters obtained for diazirine **16** and the benzophenone-containing analogues **18**<sup>22</sup> (previously reported) and **19** reveals that of those three probes containing photoactive isoprenoids the diazirine manifests the highest  $V_{\max}$  value and lowest  $K_m$  value, making it the most efficient substrate. Although the  $V_{\max}$  for **16** was lower than that for a farnesylated analogue of a-factor precursor, **17**, **16** demonstrated a lower  $K_m$  value, making the catalytic efficiency of **16** ( $V_{\max}/K_m = 143 \text{ pmol min}^{-1} \text{ mg}^{-1} \mu\text{M}^{-1}$ ) comparable to that of **17** ( $V_{\max}/K_m = 157 \text{ pmol min}^{-1} \text{ mg}^{-1} \mu\text{M}^{-1}$ ). Both of these values are higher than those obtained for benzophenone-based probes **18**<sup>22</sup> and **19** ( $V_{\max}/K_m = 52$  and  $7.0 \text{ pmol min}^{-1} \text{ mg}^{-1} \mu\text{M}^{-1}$ , respectively) suggesting that the smaller diazirine moiety is a superior mimic of an isoprene unit.

**His-Ste14p Cross-Linking with the Diazirine-Modified a-Factor.** The ability of His-Ste14p to be covalently modified with the diazirine- and benzophenone-modified a-factor peptides was assessed via photoaffinity-labeling studies (Figure 3). Purified His-Ste14p (0.25  $\mu\text{g}$ ) was incubated with 50  $\mu\text{M}$  of each peptide and irradiated with UV light (365 nm) for 30 min on ice, and the cross-linked material was resolved by SDS-PAGE. The identity of the Ste14p–a-factor conjugate was determined by three different methods: detection of the biotin moiety with NeutrAvidin HRP (Figure 3B), detection of His-Ste14 using a  $\alpha$ -Ste14p antibody (Figure 3C), and ultimately through direct fluorescence detection of the S-Fam fluorophore (Figure 3A).

Following separation of the reaction mixture by SDS-PAGE, immunoblot analysis with an  $\alpha$ -Ste14p antibody demonstrated that His-Ste14p was present in each lane at the expected molecular weight ( $\sim 36 \text{ kDa}$ ) (Figure 3C). UV-dependent photo-cross-linking of the probes to His-Ste14p was visualized using NeutrAvidin HRP. Biotinylated His-Ste14p was only present in samples subjected to UV light (Figure 3B, lanes 3, 5, and 7). The strongest signal was obtained with diazirine-containing peptide **16**, suggesting that this compound photo-labeled His-Ste14p with the highest efficiency. Additionally, UV-dependent fluorescent labeling was also observed using **16** and **19** (Figure 3A, lanes 5 and 7). Probe **18** does not contain a S-Fam moiety and hence could not be visualized in a similar



**Figure 3.** Analysis of cross-linking reactions containing purified His-Ste14p and different photoactive probes. (A) Fluorescent imaging, (B) immunoblot analysis with NeutrAvidin HRP, and (C) immunoblot analysis with  $\alpha$ -Ste14. Experiments were performed with **16**, **18**, and **19**. For this experiment, purified His-Ste14p (0.25  $\mu\text{g}$ ) was incubated with the probes indicated (50  $\mu\text{M}$ ) and irradiated on ice for 30 min followed by fractionation via SDS-PAGE. The resulting gel was visualized using (A) a fluorescence scanner or transferred to a nitrocellulose membrane and visualized using (B) NeutrAvidin HRP or (C) an  $\alpha$ -Ste14 antibody. The data shown is from one of three replicates of this experiment.

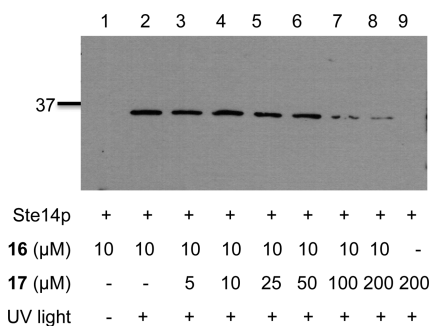
manner. Furthermore, the increased labeling efficiency with **16** observed with NeutrAvidin HRP detection was recapitulated with the fluorescence data. This increased labeling, which can be readily visualized via direct fluorescence scanning of the gel, suggests that a larger number of enzyme molecules are cross-linked. These results are in contrast to those previously reported for Rce1 with benzophenone-based probes<sup>23,29</sup> and bode well for future experiments that will be aimed at identifying the site(s) of cross-linking. Moreover, the experimental simplicity of following the cross-linking reactions via fluorescence scanning in lieu of more complicated western blotting or NeutrAvidin HRP detection should greatly facilitate optimization of the experimental conditions. Overall, the presence of both a biotin for enrichment and a fluorescent label for direct visualization should make these new probes particularly useful. In addition to the direct analysis of cross-linking reactions shown in Figure 3, pulldown experiments with **16** and **19** were also performed (see Supporting Information Figure S7) and showed that the biotin handle could be used to recover the cross-linked products from the reaction mixture.

Comparing the efficiency of cross-linking of His-Ste14p to **16**, **18**, or **19**, it is clear that the diazirine probe is the more efficient photoaffinity-labeling reagent. Because the cross-linking experiments were performed with a concentration of



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-Stel4p with **16** was examined in a competition experiment (Figure 4). A biotinylated a-factor precursor peptide, **17**, was



**Figure 4.** Competition of photolabeling of His-Stel4p by **16** using a biotinylated a-factor precursor peptide **17**. For this experiment, His-Stel4p 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-Stel4 antibody. The data shown is from one of two replicates of this experiment.

chosen as a competitor, as it was a substrate for His-Stel4p (Table 1) and closely mimicked the structure of **16** (Figure 2). Crude membranes prepared from a  $\Delta$ ste14 deletion strain overexpressing His-Stel4p were incubated with **16** in the presence or absence of increasing concentrations of the competitor **17** in the presence of UV light (Figure 4). Following incubation, the samples were enriched for biotinylated proteins via neutravidin-agarose beads, and the proteins were separated by SDS-PAGE. Immunoblot analysis with an α-Stel4p antibody revealed that the amount of cross-linked His-Stel4p decreased as the concentration of the competitor, **17**, was increased from 5 (Figure 4, lane 3) to 200 μM (Figure 4, lane 8). These data suggest that **16** is labeling the substrate binding site of His-Stel4p.

## CONCLUSIONS

Herein, we have described the development and application of a new class of isoprenoid analogue containing a photoexcitable diazirine moiety. The photoactive farnesyl mimic was prepared in six steps and incorporated into a multifunctional peptide produced via solid-phase synthesis. Kinetic analyses with His-Stel4p 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-Stel4p 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-Stel4p coupled with the ease of analysis obtained with this new class of isoprenoid mimics should facilitate the identification of active-site residues in His-Stel4p. 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-PEG<sub>4</sub>-Biotin was obtained from Thermo Scientific. The benzophenone-containing isoprenoid bromides, C<sub>10</sub>-m-Bp-Br (**2**), was prepared as previously described.<sup>15,24–27</sup> HR-ESI-MS analysis was performed using a Bio-TOF-II mass spectrometer.

**3-(3-Methyldiaziridin-3-yl)propanoic Acid (5).** Levulinic acid **4** (1.61 g, 13.8 mmol, 1 equiv) was dissolved in 7 N NH<sub>3</sub> in CH<sub>3</sub>OH (13.5 mL, 91.0 mmol, 7 equiv). The resulting solution was stirred under N<sub>2</sub> on ice for 3 h. A solution of hydroxylamine-O-sulfonic acid (1.80 g, 15.9 mmol, 1.2 equiv) in CH<sub>3</sub>OH (12 mL) was added dropwise at a rate of 1 s<sup>-1</sup>. The reaction mixture was stirred for 20 h and allowed to warm to rt. N<sub>2</sub> was bubbled through the solution for 1 h to remove NH<sub>3</sub> 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).** Diaziridine **5** was redissolved in CH<sub>3</sub>OH (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 I<sub>2</sub> 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 (2 × 20 mL). The organic layers were combined, dried over MgSO<sub>4</sub>, and concentrated to afford diazirine acid **6** as a brown residue (0.785 g, 45%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 1.05 (s, 3H), 1.73 (t, 2H, J = 6.8 Hz), 2.23 (t, 2H, J = 6.8 Hz). <sup>13</sup>C NMR (75.0 MHz, CDCl<sub>3</sub>): δ 20.4, 29.2, 30.0, 72.3, 179.1. HR-ESI-MS: calcd for C<sub>5</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub> [M–H]<sup>–</sup>, 127.0513; found, 127.0493.

**(E)-2-((3,7-Dimethylocta-2,6-dien-1-yl)oxy)tetrahydro-2H-pyran (8).** This compound was prepared via a modification of a previously described procedure.<sup>17</sup> To a solution of geraniol **7** (3.11 g, 20.2 mmol, 1 equiv) in CH<sub>2</sub>Cl<sub>2</sub> (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 overnight at rt. The reaction mixture was quenched with saturated aqueous NaHCO<sub>3</sub> and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was dried over MgSO<sub>4</sub> and concentrated to afford **8** as a clear oil (4.79 g, 99%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 1.58 (s, 3H), 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), 3.98–4.05 (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), 5.16 (t, 1H, J = 6.3 Hz), 5.39 (t, 1H, J = 6.2 Hz). HR-ESI-MS: calcd for C<sub>15</sub>H<sub>26</sub>O<sub>2</sub> [M + H]<sup>+</sup>, 239.1919; found, 239.1932.

**(2E,6E)-2,6-Dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dien-1-ol (9).** This compound was prepared via a modification of a previously described procedure.<sup>17</sup> Protected geraniol **8** was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (28 mL). In turn, 70% *t*-Bu-OOH in H<sub>2</sub>O (8.5 mL, 60.9 mmol, 3 equiv), salicylic acid (0.279 g, 2.02 mmol, 0.1 equiv), and SeO<sub>2</sub> (0.205 g, 2.03 mmol, 0.1 equiv) were added, and the resulting solution was stirred overnight at rt. The reaction mixture was quenched with saturated aqueous NaHCO<sub>3</sub>, extracted with CH<sub>2</sub>Cl<sub>2</sub>,

and dried over  $\text{MgSO}_4$ . After concentration, the residue was purified by flash chromatography (hexanes/ $\text{Et}_2\text{O}$ , 3:2, v/v) on silica gel to obtain 2.36 g (46%) of alcohol **9** as a clear oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  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), 5.16 (t, 1H,  $J$  = 6.3 Hz), 5.39 (t, 1H,  $J$  = 6.2 Hz). HR-ESI-MS: calcd for  $\text{C}_{15}\text{H}_{26}\text{O}_3\text{Na}$  [ $M + \text{Na}$ ] $^+$ , 277.1790; found, 277.1763.

**(2E,6E)-2,6-Dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dien-1-yl 3-(3-Methyl-3H-diazirin-3-yl)propanoate (10).** To a solution of protected alcohol **9** (1.94 g, 7.60 mmol, 1 equiv) and DMAP (92.8 mg, 0.76 mmol, 0.1 equiv) in  $\text{CH}_2\text{Cl}_2$  (7 mL) was added a solution of the diazirine acid **6** (0.97 g, 7.60 mmol, 1 equiv) in  $\text{CH}_2\text{Cl}_2$  (10 mL). DIC (0.959 g, 7.60 mmol, 1 equiv) was added, and the resulting solution was stirred at rt for 16 h. The reaction mixture was filtered and concentrated. Purification by flash chromatography (hexanes/ $\text{EtOAc}$ , 5:1) afforded 1.07 g (57%) of diazirine **6** as a clear oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.03 (s, 3H), 1.48–1.85 (m, 14H), 2.04–2.22 (m, 6H), 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,  $J$  = 6.3, 12 Hz), 4.46 (s, 2H), 4.62 (t, 1H,  $J$  = 2.7 Hz), 5.36 (t, 1H,  $J$  = 6.3 Hz), 5.44 (t, 1H,  $J$  = 6.2 Hz).  $^{13}\text{C}$  NMR (75.0 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.16, 16.4, 20.5, 23.7, 25.1, 26.6, 30.6, 32.9, 39.7, 44.2, 63.3, 65.6, 69.3, 72.3, 105.5, 118.5, 130.4, 130.7, 141.8, 173.1. IR (NaCl,  $\text{cm}^{-1}$ ): 2940 (s), 2870 (m), 1737 (s), 1669 (w), 1587 (w), 1447 (w), 1385 (m), 1175 (m), 1117 (m), 1023 (m). HR-ESI-MS: calcd for  $\text{C}_{20}\text{H}_{32}\text{N}_2\text{O}_4\text{Na}$  [ $M + \text{Na}$ ] $^+$ , 387.2260; found, 387.2234.

**(2E,6E)-8-Hydroxy-2,6-dimethylocta-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  $\text{EtOH}$  (1.60 mL) was added PPTS (6.81 mg, 271  $\mu\text{mol}$ , 0.1 equiv). The reaction flask was fitted with a septum and stirred at 60  $^\circ\text{C}$  for 4 h. The reaction mixture was concentrated in vacuo and purified by flash chromatography (hexanes/ $\text{EtOAc}$ , 2:1) to afford 75 mg (99%) of diazirine alcohol **11** as a clear oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.03 (s, 3H), 1.58–1.75 (m, 8H), 2.05–2.22 (m, 6H), 4.15 (t, 2H,  $J$  = 6.0 Hz), 4.47 (s, 2H), 5.38–5.45 (m, 2H).  $^{13}\text{C}$  NMR (75.0 MHz,  $\text{CDCl}_3$ ):  $\delta$  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,  $\text{cm}^{-1}$ ): 3395 (br), 2925 (s), 2870 (m), 1735 (s), 1669 (w), 1587 (w), 1447 (m), 1385 (m), 1176 (m), 1000 (m). HR-ESI-MS: calcd for  $\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}_3\text{Na}$  [ $M + \text{Na}$ ] $^+$ , 303.1685; found, 303.1659.

**(2E,6E)-8-Bromo-2,6-dimethylocta-2,6-dien-1-yl 3-(3-Methyl-3H-diazirin-3-yl)propanoate (12).** Diazirine alcohol **11** (69.5 mg, 0.247 mmol, 1 equiv) was converted to the corresponding bromide in the presence of resin-bound  $\text{PPh}_3$  (250 mg, 1.08 mmol, 4 equiv) and  $\text{CBr}_4$  (350 mg, 1.08 mmol, 4 equiv) dissolved in  $\text{CHCl}_3$  (6 mL). The resulting solution was allowed to stir for 2 h at rt. After the reaction was complete, excess  $\text{CBr}_4$  and resin-bound  $\text{PPh}_3$  were removed from the mixture by passing the reaction through a  $\text{C}_{18}$  Sep-Pak column. Removal of the solvent afforded 68.1 mg (80%) of diazirine bromide **12** as a clear oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.03 (s, 3H), 1.58–1.75 (m, 8H), 2.05–2.22 (m, 6H), 3.99 (d, 2H,  $J$  = 8.4 Hz), 4.47 (s, 2H), 5.39 (t, 1H,  $J$  = 6 Hz), 5.51 (t, 2H,  $J$  = 6.1 Hz).  $^{13}\text{C}$  NMR (75.0 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.6, 15.4, 23.5, 26.4, 30.8, 32.9, 38.3, 44.2, 69.3, 72.1, 123.3, 130.4, 130.7, 143.2, 173.1. IR (NaCl,  $\text{cm}^{-1}$ ): 2925 (m), 2870 (m), 1736 (s), 1656 (w), 1586 (w), 1446 (m), 1385 (m), 1173 (m). HR-ESI-MS: calcd for  $\text{C}_{15}\text{H}_{23}\text{BrN}_2\text{O}_2\text{Na}$  [ $M + \text{Na}$ ] $^+$ , 365.0840 ( $^{79}\text{Br}$ ); found, 365.0862; 367.0819, ( $^{81}\text{Br}$ ); found, 367.0831.

**Synthesis of Biotin-Peg<sub>4</sub>-K(5-Fam)YIIKGVFWDPAC-OH (15).** 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 on preloaded Fmoc-Cys(Trt)-Wang resin (0.25 mmol), and the peptide chain was elongated using HCTU/*N*-methylmorpholine-catalyzed, single coupling steps with 4 equiv of both protected amino acids and HCTU for 30 min. 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. The resin containing the peptide

was washed with  $\text{CH}_2\text{Cl}_2$ , dried in vacuo overnight, weighed, and divided into three portions for further synthesis on a reduced scale. Using 83.0  $\mu\text{mol}$  of peptide, the free amino terminus was biotinylated in DMF (5 mL) with NHS-PEG<sub>4</sub>-Biotin (0.49 mg, 83.0  $\mu\text{mol}$ , 1 equiv) catalyzed by DIEA (14.4  $\mu\text{L}$ , 8.3  $\mu\text{mol}$ , 0.1 equiv) for 16 h. After acylation, the resin-bound peptide was washed thoroughly with  $\text{CH}_2\text{Cl}_2$  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 Dde-protected side chain. After verifying the deprotection was complete by ninhydrin analysis, the peptide was washed with  $\text{CH}_2\text{Cl}_2$ , dried, and then reacted 5-FAM SE (45 mg, 86.0  $\mu\text{mol}$ , 1 equiv) catalyzed by DIEA (14.4  $\mu\text{L}$ , 8.3  $\mu\text{mol}$ , 0.1 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-ethanedithiol (0.25 mL), thioanisole (0.5 mL), and  $\text{H}_2\text{O}$  (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  $\text{Et}_2\text{O}$  (100 mL). The crude solid peptide was collected by centrifugation, the supernatant was removed, and the resulting pellet was washed two times with cold  $\text{Et}_2\text{O}$  (50 mL), repeating the centrifugation and supernatant-removal steps each time. The crude peptide was purified using a semipreparative  $\text{C}_{18}$  RP-HPLC column with detection at 280 nm and eluted with a gradient of solvent A ( $\text{H}_2\text{O}$ /0.1% TFA, v/v) and solvent B ( $\text{CH}_3\text{CN}$ /0.1% TFA, v/v). The crude peptide (150 mg) was dissolved in a DMF/ $\text{H}_2\text{O}$  solution (1:5 v/v, 25 mL), 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  $\text{C}_{18}$  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 and concentrated by lyophilization to yield 55 mg (37% yield) of a yellow solid. A small amount (<1 mg) of the resulting purified peptide was dissolved in 10  $\mu\text{L}$  of 0.1% TFA/ $\text{CH}_3\text{CN}$  and diluted 1:50 in a mixture of  $\text{CH}_3\text{CN}$ / $\text{H}_2\text{O}$  (1:1 v/v) prior to MS analysis. MS was performed using a 50  $\mu\text{L}$  injection and collecting 3000 scans. ESI-MS: calcd for  $\text{C}_{117}\text{H}_{155}\text{N}_{19}\text{O}_{30}\text{S}_2$  [ $M + 2\text{H}$ ] $^{+2}$ , 1186.0334; found, 1186.0453.

**Synthesis of Biotin-Peg<sub>4</sub>-K(5-Fam)YIIKGVFWDPAC(C10-Diazirine)-OH (16).** Peptide **15** (20 mg, 5.3  $\mu\text{mol}$ , 1 equiv) was dissolved in DMF/*n*-butanol/ $\text{H}_2\text{O}$  (0.10% TFA) (3:1:1, v/v/v, 6 mL). Isoprenoid bromide **12** (15 mg, 54.5  $\mu\text{mol}$ , 10 equiv) was dissolved in 0.50 mL of DMF and loaded onto a  $\text{C}_{18}$  Sep-Pak column that had been equilibrated with 5%  $\text{CH}_3\text{CN}$  in aqueous 0.10% TFA. The column was washed with 5%  $\text{CH}_3\text{CN}$  (5 mL) followed by 30%  $\text{CH}_3\text{CN}$  (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.  $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$  (5.4 mg, 25  $\mu\text{mol}$ , 5 equiv) was then added to initiate the alkylation reaction. After 4 h, the reaction was analyzed by analytical RP-HPLC, purified by semipreparative  $\text{C}_{18}$  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  $\text{C}_{132}\text{H}_{177}\text{N}_{21}\text{O}_{32}\text{S}_2$  [ $M + 2\text{H}$ ] $^{+2}$ , 1317.1223; found, 1317.1172.

**Synthesis of Biotin-Peg<sub>4</sub>-YIIKGVFWDPAC(Fr)-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  $\mu\text{mol}$  of peptide, the free amino terminus was biotinylated in DMF (5 mL) with NHS-PEG<sub>4</sub>-Biotin (0.49 mg, 83.0  $\mu\text{mol}$ , 1 equiv) catalyzed by DIEA (14.4  $\mu\text{L}$ , 8.3  $\mu\text{mol}$ , 0.1 equiv) for 16 h. After Reagent K cleavage and HPLC purification, the free thiol-containing peptide (20 mg, 13  $\mu\text{mol}$ , 1 equiv) was prenylated with farnesyl bromide (10 mg, 65  $\mu\text{mol}$ , 5 equiv) using the same conditions as **16**. Purity by HPLC: 92.4%. ESI-MS: calcd for  $\text{C}_{105}\text{H}_{157}\text{N}_{17}\text{O}_{23}\text{S}_2$  [ $M + 2\text{H}$ ] $^{+2}$ , 1045.0551; found, 1045.0499.

**Synthesis of Biotin-Peg<sub>4</sub>-K(5-Fam)YIIKGVFWDPAC(C5-m-BP)-OH (19).** Starting with **15**, as previously described, the peptide was prenylated using the same conditions and quantities as used for **16**



with replacement of the isoprenoid analogue **12** with  $C_5$ -*m*-Bp-Br that was prepared as previously described.<sup>19,21</sup> Purity by HPLC: 94.2%. ESI-MS: calcd for  $C_{136}H_{173}N_{19}O_{32}S_2$  [ $M + 2H$ ]<sup>2+</sup>, 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.<sup>7,30</sup> 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  $N_2$ , and stored at  $-80^\circ C$ . In vitro assays for methyltransferase activity were performed using crude membranes as previously described.<sup>22</sup> In brief, reactions contained crude membrane preparations, 200  $\mu M$  AFC, 20  $\mu M$  S-adenosyl-L-[methyl- $^{14}C$ ]methionine ([ $^{14}C$ ]SAM) (50–60 mCi/mmol), and 100 mM Tris-HCl, pH 7.5, in 60  $\mu L$ . The reactions were incubated at  $30^\circ C$  for 30 min and terminated by the addition of 50  $\mu L$  of 1 M NaOH and 1% SDS (v/v). Each reaction (100  $\mu L$ ) 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 diffuse at rt for 2.5 h. The filters were discarded, and the base-labile [ $^{14}C$ ]methyl groups transferred were measured via liquid scintillation counting. For experiments performed with purified His-Ste14p, His<sub>10</sub>myc<sub>3</sub>N-Ste14p (His-Ste14p) was expressed in a  $\Delta ste14$  deletion strain<sup>30</sup> and purified as previously described.<sup>7</sup>

**Photo-Cross-Linking and Neutravidin-Agarose Pulldown Assays in Crude Membranes.** Photo-cross-linking assays were performed as described previously, with minor modifications.<sup>20,22</sup> 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  $4^\circ C$  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  $\mu L$  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  $\mu L$  of a 50% neutravidin/RIPA bead slurry for 2 h at  $4^\circ C$ . 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  $\mu L$  of 2 $\times$  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  $65^\circ C$  and subjected to 12% SDS-PAGE and immunoblot analysis.

**Photo-Cross-Linking of Purified His-Ste14p.** Purified His-Ste14p (5  $\mu g$ ) in 138 mM MOPS and 1 mM DTT were incubated in the presence of the photoaffinity analogues and incubated at  $4^\circ C$  for 10 min. The samples were irradiated (365 nm) in 96-well plates for 30 min on ice. Following photo-cross-linking, 40  $\mu L$  of 5 $\times$  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)) was added directly to the samples. Samples were heated for 30 min at  $65^\circ C$  and subjected to 12% SDS-PAGE.

**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 band-pass emission filter (520 BP 40). The images were collected with a pixel size of 50  $\mu m$  at normal sensitivity with a PMT voltage of 400 V.

**Immunoblot Analysis.** Proteins from SDS-PAGE gels were transferred to nitrocellulose membranes (0.22  $\mu m$ ), 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  $Na_2HPO_4$ , 1.8 mM  $KH_2PO_4$ , and 0.05% (v/v) Tween-20, pH 7.4) (PBST). The blocked membrane was incubated for 2 h at rt with an  $\alpha$ -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  $\alpha$ -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,  $^{13}C$  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>.

## ■ AUTHOR INFORMATION

### Corresponding Authors

\*E-mail: Hrycyna@purdue.edu (C.A.H.).

\*E-mail: Diste001@umn.edu (M.D.D.).

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This research was supported by funds from the NIH (grants R01 GM084152 to M.D.D. and R01 GM106082 to C.A.H.).

## ■ REFERENCES

- (1) Vervacke, J. S.; Wang, Y.-C.; Distefano, M. D. *Curr. Med. Chem.* **2013**, *20*, 1585–1594.
- (2) Zhang, F. L.; Casey, P. J. *Annu. Rev. Biochem.* **1996**, *65*, 241–269.
- (3) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*, 4th ed.; W.H. Freeman: New York, 2008.
- (4) McTaggart, S. J. *Cell. Mol. Life Sci.* **2006**, *63*, 255–267.
- (5) Griggs, A. M.; Hahne, K.; Hrycyna, C. A. *J. Biol. Chem.* **2010**, *285*, 13380–13387.
- (6) Ashby, M. N. *Curr. Opin. Lipidol.* **1998**, *9*, 99–102.
- (7) Anderson, J. L.; Frase, H.; Michaelis, S.; Hrycyna, C. A. *J. Biol. Chem.* **2005**, *280*, 7336–7345.
- (8) Manandhar, S. P.; Hildebrandt, E. R.; Jacobsen, W. H.; Santangelo, G. M.; Schmidt, W. K. *Yeast* **2010**, *27*, 327–343.
- (9) Berndt, N.; Hamilton, A. D.; Sebt, S. Ø. M. *Nat. Rev. Cancer* **2011**, *11*, 775–791.
- (10) Ochocki, J.; Distefano, M. D. *MedChemComm* **2013**, *4*, 476–492.
- (11) 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*, 8206–8219.
- (12) Baba, T.; Allen, C. M. *Biochemistry* **1984**, *23*, 1312–1322.
- (13) Bikhtiyarov, Y. E.; Omer, C. A.; Allen, C. M. *J. Biol. Chem.* **1995**, *270*, 19035–19040.
- (14) Hovlid, M. L.; Edelstein, R. L.; Henry, O.; Ochocki, J.; Talbot, T.; Lopez-Gallego, F.; Schmidt-Dannert, C.; Distefano, M. D. *Chem. Biol. Drug Des.* **2010**, *76*, 107–115.
- (15) Yokoyama, K.; McGeady, P.; Gelb, M. H. *Biochemistry* **1995**, *34*, 1344–1354.
- (16) Alexander, M.; Gerauer, M.; Pechlivanis, M.; Popkova, B.; Dvorsky, R.; Brunsveld, L.; Waldmann, H.; Kuhlmann, J. *ChemBioChem* **2009**, *10*, 98–108.
- (17) DeGraw, A. J.; Zhao, Z.; Strickland, C. L.; Taban, A. H.; Hsieh, J.; Jefferies, M.; Xie, W.; Shintani, D. K.; McMahan, C. M.; Cornish, K.; Distefano, M. D. *J. Org. Chem.* **2007**, *72*, 4587–4595.
- (18) Gaon, I.; Turek, T. C.; Weller, V. A.; Edelstein, R. L.; Singh, S. K.; Distefano, M. D. *J. Org. Chem.* **1996**, *37*, 4845–4848.
- (19) Turek, T. C.; Gaon, I.; Distefano, M. D.; Strickland, C. L. *J. Org. Chem.* **2001**, *66*, 3253–3264.
- (20) Turek-Etienne, T. C.; Strickland, C. L.; Distefano, M. D. *Biochemistry* **2003**, *42*, 3716–3724.
- (21) Kale, T. A.; Raab, C.; Yu, N.; Dean, D. C.; Distefano, M. D. *J. Am. Chem. Soc.* **2001**, *123*, 4373–4381.

- (22) Hahne, K.; Vervacke, J. S.; Shrestha, L.; Donelson, J. L.; Gibbs, R. A.; Distefano, M. D.; Hrycyna, C. A. *Biochem. Biophys. Res. Commun.* **2012**, 423, 98–103.
- (23) Kyro, K.; Manandhar, S. P.; Mullen, D.; Schmidt, W. K.; Distefano, M. D. *Bioorg. Med. Chem.* **2010**, 18, 5675–5684.
- (24) Chowdhry, V.; Vaughan, R.; Westheimer, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, 73, 1406–1408.
- (25) Michaelis, S.; Barrowman, J. *Microbiol. Mol. Biol. Rev.* **2012**, 76, 626–651.
- (26) Bond, M. R.; Zhang, H.; Vu, P. D.; Kohler, J. J. *Nat. Protoc.* **2009**, 4, 1044–1063.
- (27) Atherton, E.; Hardy, P. M.; Harris, D. E.; Matthews, B. H. In *Peptides 1990*; Giralt, E., Andreu, D., Eds.; ESCOM: Leiden, The Netherlands, 1991.
- (28) Anderegg, R. J.; Betz, R.; Carr, S. A.; Crabb, J. W.; Duntze, W. J. *Biol. Chem.* **1988**, 263, 18236–18240.
- (29) Kyro, K.; Manandhar, S. P.; Mullen, D.; Schmidt, W. K.; Distefano, M. D. *Bioorg. Med. Chem.* **2011**, 19, 7559–7569.
- (30) Anderson, J. L.; Henriksen, B. S.; Gibbs, R. A.; Hrycyna, C. A. *J. Biol. Chem.* **2005**, 280, 29454–29461.