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Photoaffinity labeling of Ras converting enzyme 1 (Rce1p) using a benzophenone-containing peptide substrate

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ABSTRACT

Isoprenylation is a post-translational modification that increases protein hydrophobicity and helps target certain proteins to membranes. Ras converting enzyme 1 (Rce1p) is an endoprotease that catalyzes the removal of a three residue fragment from the C-terminus of isoprenylated proteins. To obtain structural information about this membrane protein, photoaffinity labeling agents are being prepared and employed. Here, we describe the synthesis of a benzophenone-containing peptide substrate analogue for Rce1p. Using a continuous spectrofluorometric assay, this peptide was shown to be a substrate for Rce1p. Mass spectrometry was performed to confirm the site of cleavage and structure of the processed probe. Photolysis of the biotinylated compound in the presence of membranes containing Rce1p followed by streptavidin pull-down and Western blot analysis indicated that Rce1p had been labeled by the probe. Photolysis in the presence of both the biotinylated, benzophenone-containing probe and a farnesylated peptide competitor reduced the extent of labeling, suggesting that labeling is occurring in the active site.

1. Introduction

1.1. Sequential processing of *ras* proteins

Protein isoprenylation, proteolysis, and carboxylmethylation are sequential post-translational modifications that occur to *ras* proteins. These modifications increase protein hydrophobicity, assist in membrane localization, and facilitate signal transduction.[1–4] *Ras* is a GTP-binding protein (G-protein) that contains a Ca_1a_2X motif. The post-translational processing of Ca_1a_2X proteins (Fig. 1) first involves isoprenylation at the cysteine residue of the Ca_1a_2X sequence by either protein farnesyltransferase (PFTase) or protein geranylgeranyl transferase (PGGTase). The -X residue of the Ca_1a_2X sequence influences the identity of the isoprenoid, and $-a_1a_2$ are typically aliphatic amino acids.[2,3,5] Ca_1a_2X-containing isoprenylated proteins are then modified at the C-terminus by the

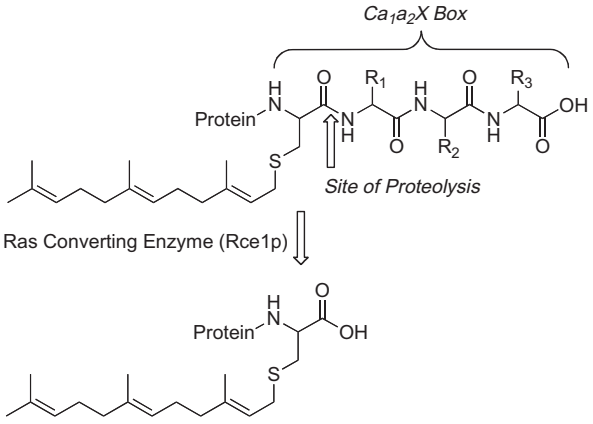

Figure 1. Rce1p proteolysis of the last three residues of a C-terminal tetrapeptide Ca_1a_2X motif. C, cysteine; R_1R_2, any aliphatic amino acid; R_3, one of several amino acids.

Abbreviations: Abz, 2-aminobenzoyl; C5BP, 3-methylbenzophenone prenyl ether; CLEAR, crosslinked ethoxylate acrylate resin; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene) ethyl; DI, deionized; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dnp, dinitrophenyl; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; ESI-MS, electrospray ionization mass spectrometry; FA, formic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; Fr, farnesyl; HA, hemagglutinin; HRP, horseradish peroxidase; BME, 2-mercaptoethanol; OAc, acetate; PBS, phosphate buffered saline; PEG, polyethylene glycol; PVDF, polyvinylidene difluoride; PMSF, phenylmethanesulfonyl fluoride; RIPA, radioimmunoprecipitation assay; RP-HPLC, reversed-phase high pressure liquid chromatography; SA, streptavidin; SPPS, solid-phase peptide synthesis; TBST, Tris-buffered saline containing Tween-20; TFA, trifluoroacetic acid; TFP, tetrafluorophenyl.

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protease, Ras converting enzyme 1 (Rce1p), which removes the -a_1a_2X tripeptide in the endoproteolysis step (Fig. 1). This is followed by carboxylmethylation of the C-terminal prenyl cysteine by the isoprenylcysteine carboxymethyltransferase (Icmt), also called Ste14p in yeast.[1,2,4–8]

The mechanism of catalysis for Rce1p remains undefined, partly because its membrane association makes it difficult to purify. Rce1p localizes at the ER and is predicted to have multiple membrane spanning regions.[1,8,9] The critical catalytic residues of Rce1p are purported to be embedded in the membrane to facilitate interaction with isoprenoid-modified substrates, but the structure and topology of Rce1p have yet to be resolved.[10] Rce1p is involved in the membrane localization of all known forms of human *ras* and inhibition of its protease activity alters *ras* function.[5] Mutated forms of *ras* proteins are associated with cancerous signal transduction in roughly 15–20% of human cancers. Consequently, disruption of Ca_1a_2X proteolysis is viewed as an important therapeutic strategy in targeting *ras*-mediated cancers.[1,4]

Mutation, inhibition, and bioinformatic studies have been performed to determine the mechanism and protease class of Rce1p. Independent studies suggest that Rce1p is either a metalloprotease or a cysteine protease, but it is still possible that Rce1p belongs to a yet undiscovered class of proteases.[1,8,10–12] The cysteine protease classification for Rce1p has been questioned by mutational and bioinformatic investigation.[8,10,12] Even though Rce1p is reported to require a conserved cysteine (Cys^{251}) for activity,[1] experimental analyses maintain that Cys^{251} is not required for Rce1p function.[8,10,12]

Sequential analysis of Rce1p identified invariant residues reminiscent of a consensus HEXXH motif found in metalloproteases.[1,10,12] Metal-dependent enzymes require two histidine residues and at least one glutamate residue for activity.[10] Site-directed mutagenesis revealed that three conserved residues (Glu^{156}, His^{194}, and His^{248}) were required for Rce1p activity.[1,10] The confirmation that Rce1p was inhibited by 1,10-phenanthroline and excess zinc offered additional support for the metal-dependent protease mechanism.[8,10] The evidence that Rce1p proteolysis requires histidine and glutamate residues does not, however, rule out the possibility that Rce1p utilizes a yet undiscovered protease mechanism.

To begin to address the issue that currently, no structural information exists for Rce1p,[10] we have designed substrate analogues that incorporate photoactive isoprenoids that can potentially be used to label and identify active site residues.

1.2. Design of Rce1p substrate analogue incorporating a benzophenone photophore into the prenyl group

Isoprenylated peptide substrate, **2** (Fig. 2), derived from the C-terminal sequence (CVIM) of mammalian K-Ras4B, has been an effective substrate in Rce1p proteolysis assays.[13] This farnesylated, nona-peptide contains an N-terminal Abz (2-aminobenzoyl) fluorophore and a dinitrophenyl quencher (lysine ε-dinitrophenyl) is attached at the a_1 (V) position.[8] It has been previously reported that both KSKTKC(Fr)VIM and Abz-KSKTKC(Fr)VIM are equally good substrates for Rce1p, exhibiting identical kinetic parameters (K_M = 4 μM; V_{max} = 600 nmol/h/mg Rce1p-containing membrane protein) and indicating that the fluorophore has no effect on peptide proteolysis.[9,10,13] V_{max} decreased by half (V_{max} = 300 nmol/h/mg Rce1p-containing membrane protein) when the quencher was linked at the V position, but the peptide was still an efficient substrate.[13]

Compound **4** (Fig. 3) is an analogue of **2**, containing a photoactive benzophenone-modified isoprenoid linked to the peptide. The

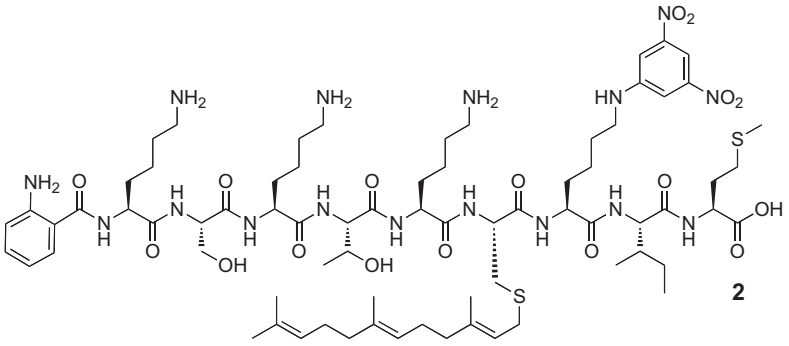

Figure 2. Structure of isoprenylated peptide substrate, **2**, derived from the C-terminal sequence (CVIM) of mammalian K-Ras4B.

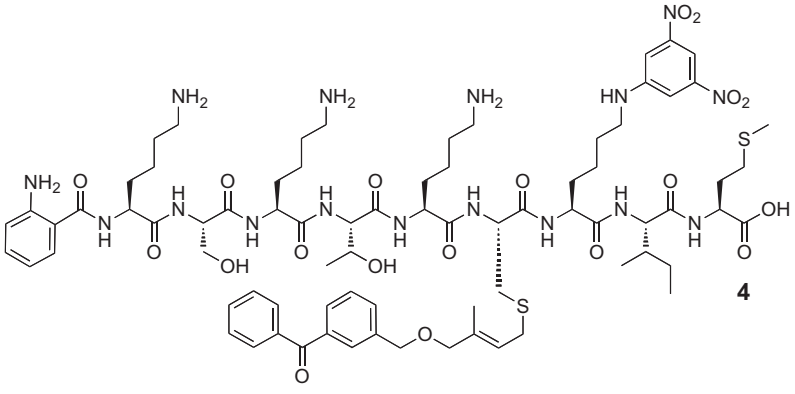

Figure 3. Structure of substrate analogue, **4**, incorporating a benzophenone-modified isoprenoid.

benzophenone group is tethered to the isoprenoidal portion of the probe by a stable ether bond to permit photocrosslinking of Rce1p.[7,14,15]

Benzophenone photophores have proven efficacy as monodentate photoaffinity labeling tools.[16] They combine stability with specificity and allow enough photoincorporation for product identification.[17] The aryl ketone probes can be crosslinked to membranes in order to map their hydrophobic surface.[7] The isoprenoid-linked aryl ketone photophore (Fig. 3) bears enough structural similarity to a C15 isoprenoid to possibly be useful for protein incorporation.[18,19] While other types of photoactive isoprenoids have been prepared including diazo esters[20–25] and aryl azides,[26] we chose to employ the benzophenone-based structures due to their ease of synthesis and their ability to be excited at wavelengths above 300 nm. It should be noted that while a plethora of modified isoprenoid diphosphates have been used to study prenyltransferases and interactions between prenylated proteins and other proteins,[27,28] the use of photoactive isoprenoid analogues to probe the processing enzyme, Rce1p, remains a largely unexplored approach. Here we describe the utility of one such molecule and show that it is an alternative substrate for Rce1p. Irradiation of this probe in the presence of partially-purified membranes containing Rce1p results in selective labeling of the target enzyme.

2. Results and discussion

2.1. Synthesis of Rce1p substrates

Peptide **1** (Fig. 4) was synthesized by Fmoc-based solid-phase peptide synthesis prior to alkylation with farnesyl bromide, using Zn^{2+} catalysis under acidic conditions to create peptide **2** (Fig. 2).[29] probe **4** (Fig. 3) was prepared in a similar fashion using bromide **3** (Fig. 4).[18,20]

Peptides were purified by RP-HPLC and characterized by mass spectrometry. ESI-MS–MS of spectra of **2** and **4** are shown in Figure 5. For peptide **2**, the most abundant $[M+3H]^{3+}$ species (m/z = 519.4) was fragmented to yield a large number of a-, b-, and y-type fragments; four out of nine a-type ions, six out of nine b-type ions, and six out of nine y-type ions were observed, all consistent with the proposed structure of **2** (see Table 2 in the Supplementary data for complete listing and assignments). Interestingly, a large number of fragments where loss of the farnesyl group had occurred were observed. The most abundant ion in the ESI-MS–MS was the doubly-charged parent ion that had lost the farnesyl group (m/z = 676.3) (Fig. 5A). For peptide **4**, similar results were observed. Fragmentation of the $[M+3H]^{3+}$ species resulted in the production of a number of a-, b-, and y-type ions; four out of nine b-type ions and six out of nine y-type ions were observed (see Table 3 in the Supplementary data), all consistent with the desired peptide **4**. As was noted with **2**, a large number of ions where loss of the benzophenone-isoprenoid moiety had occurred were observed; those include ions related to the doubly and triply charged parent ions (m/z = 709.5 and m/z = 473.3) (Fig. 5B). A very intense ion from the benzophenone fragment (BP) (m/z = 195.1) was also observed. This fragmentation may be useful in identifying specific cross-linked peptides in future MS–MS analysis.

For photolabeling applications and subsequent pull-down experiments, the biotinylated peptide **6** (Fig. 6) was synthesized. This was accomplished by first preparing **5** (Fig. 6), which contains a biotin group linked to the peptide N-terminus via a PEG spacer. Peptide **5** also contains a orthogonally-protected lysine (using a Dde group) to allow for the introduction of a fluorophore in future experiments. Peptide **6** was obtained by alkylation of **5** with **3** (Fig. 4) using conditions analogous to those noted above.

2.2. Fluorescence-based proteolysis assay for kinetic analysis of farnesylated and benzophenone-modified probes

A previously-described, fluorescence-based proteolysis assay has been used to evaluate proteolytic processing of prenylated peptide substrate **2**.[9,13] Cleavage of the C-terminal -K(Dnp)IM tripeptide results in a time-dependent increase in fluorescence of the Abz fluorophore (420 nm) that is used to monitor the rate of endoproteolysis. This fluorogenic assay was used to evaluate **4** as an alternative substrate for Rce1p. For comparative purposes, assays were also performed with **2**.[1,8,9,13]

Carbonate-washed membranes enriched for Rce1p were isolated from yeast over-expressing Rce1p as the sole Ca_1a_2X protease; the structurally unrelated Ste24p/Afc1p protease cleaves certain Ca_1a_2X sequences, but not those in Ras-based substrates.[5] The Rce1p-enriched membranes were added to a solution containing the peptide substrates; a fluorescence plate reader was used to monitor Rce1p activity. Kinetic evaluation demonstrated that **4** is a Rce1p substrate with a K_M of 1.9 μM and a V_{max} of 0.093 nmol/min/mg protein (Table 1, Fig. 7B). Comparison of those values with analogous kinetic parameters obtained with **2** (Table 1, Fig. 7A) suggests that **4** binds to Rce1p with higher affinity (3.4-fold) than **2** but that the maximal rate is approximately 60-fold lower. For

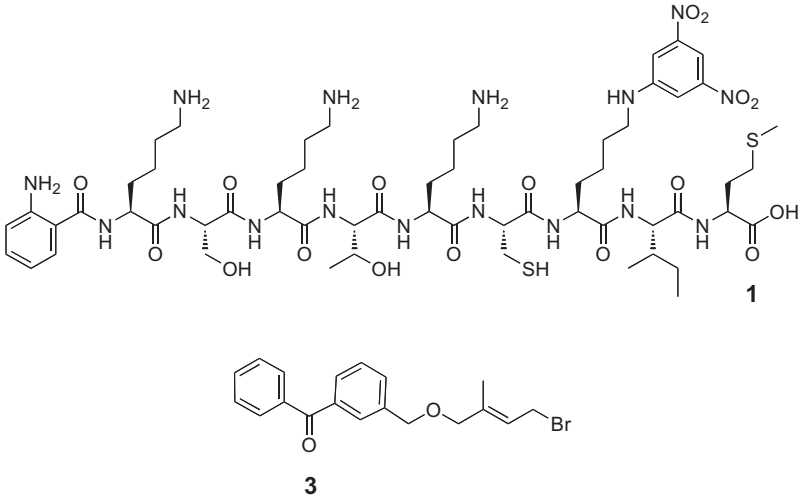

Figure 4. Structures of precursor compounds **1** and **3**. Peptide **1**, containing a free cysteine thiol, was alkylated with farnesyl bromide to produce peptide **2**. The benzophenone-modified isoprenoid, **3**, was used for alkylation of **1** to produce the aryl ketone-containing peptide photophore, **4**.

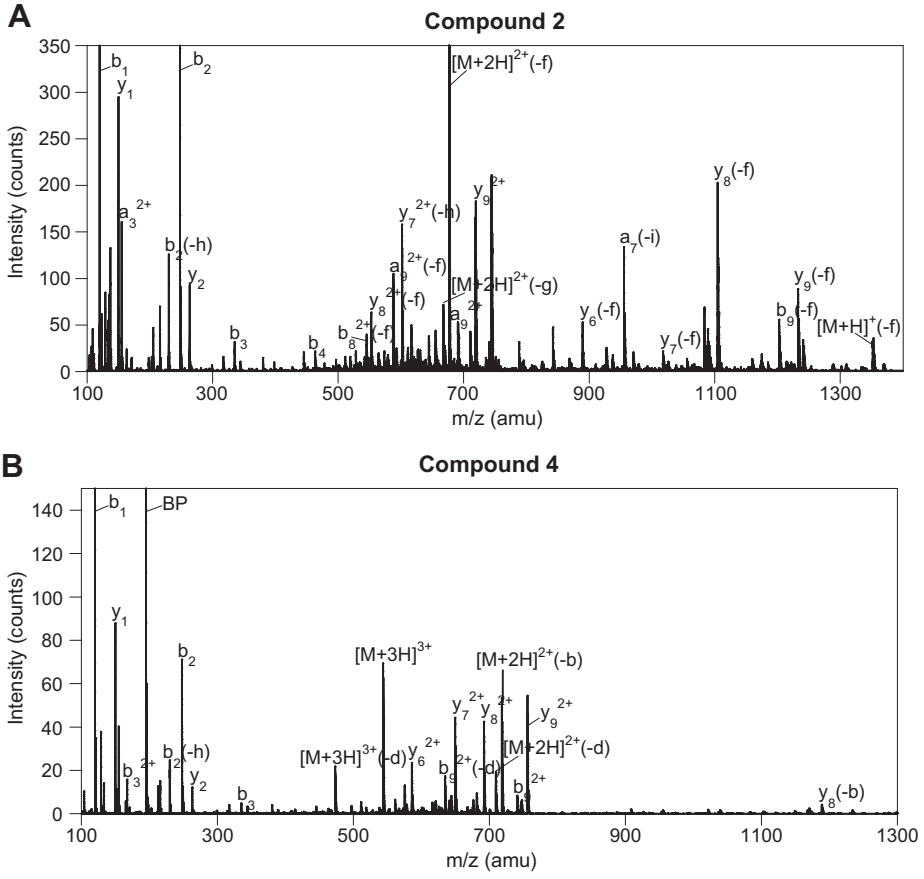

Figure 5. ESI-MS–MS of Rce1p substrates. (A) Farnesylated substrate **2** (-f, loss of farnesyl ($C_{15}H_{25}$); -g, loss of farnesyl-H_2O ($C_{15}H_{27}O$); -h, loss of H_2O; -i, loss of NH_3). (B) Benzophenone-modified substrate **4** (BP, benzophenone fragment, (3-benzoylphenyl)methylium ($C_{14}H_{11}O^+$); -b, loss of benzophenone fragment; -d, loss of benzophenone–H_2O, (3-(hydroxymethyl)phenyl)(phenyl)methanone ($C_{14}H_{13}O_2$); -h, loss of H_2O).

several reasons, the value for V_{max} for **2** differs from that previously reported.[8,30] First, there is the impact of curve fitting to the Hill equation instead of the Michaelis–Menten equation as done previously; the Hill equation provides a better fit for these and past data. Second, V_{max} is reported per mg of membrane protein, whereas previous values were not adjusted in this manner. Identical analysis of previous data (i.e., Hill equation and normalization to mg of protein) yields V_{max} values approximately half that reported here (2.5 and 2.8 vs 5.6 nmol/min/mg). We attribute the higher V_{max} value in this study to the use of membranes better enriched for Rce1p activity (i.e., carbonate-washed membranes).

2.3. Mass spectrometric analysis of enzymatic processing of farnesylated and benzophenone-modified probes

To confirm the identity of the product obtained from Rce1p-catalyzed proteolysis of **4**, a large-scale (500 μL) incubation of **4** with Rce1p was performed. Soluble products were purified using reversed phase chromatography and analyzed by ESI-MS. A similar large-scale reaction and analysis was carried out with **2** to compare the MS fragmentation. Direct infusion of the products obtained from Rce1p-catalyzed proteolysis of **2** revealed that the most abundant $[M+2H]^{2+}$ species (m/z = 509.4) in the ESI-MS was consistent with the expected product **7** (Fig. 8). Fragmentation of that ion via MS–MS (Fig. 9A) resulted in the formation of a-, b-, and y-type ions, as was observed with **2**; in total, one out of six a-type ions, four out of six b-type ions, and one out of six y-type ions, were observed. Cleavage of the thioether bond linking the farnesyl group to the peptide was a common mode of fragmentation. Similar results were obtained with the product derived from Rce1p-catalyzed proteolysis of **4**. The dominant $[M+2H]^{2+}$ species (m/z = 546.4) in the ESI-MS is consistent with the expected product **8** (Fig. 8). MS–MS fragmentation of that ion (Fig. 9B) produced a full set of both b- and y-type ions all consistent with the proposed structure for **8**. As was noted for **7**, cleavage of the thioether bond linking the isoprenoid moiety to the cysteinyl thiol constitutes a major manifold of fragmentation in these compounds.

2.4. Photoaffinity labeling of Rce1p using biotinylated, photoactive peptide substrate 6

With the confirmation that Rce1p can act on a substrate containing a photoactive isoprenoid, the related peptide **6** that incorporates a biotin moiety was employed in photoaffinity labeling experiments. A carbonate-washed membrane fraction from *Saccharomyces cerevisiae* was used as the source for Rce1p (*RCE1*). A similar membrane fraction isolated from a strain lacking Rce1p (*rce1*Δ) was employed for negative control experiments; the strains also lack Ste24p in both cases. Photolysis reactions were conducted at 4 °C using **6** at saturating concentrations.[19,20,31] The membrane protein fractions were irradiated, concentrated by ultracentrifugation, and treated with streptavidin beads to capture any cross-linked proteins. After elution, the resulting proteins were fractionated by SDS–PAGE and analyzed by Western blotting using antibodies directed against HA, an epitope tag incorporated into Rce1p via recombinant methods.[10]

Irradiation of **6** in the presence of membranes containing Rce1p resulted in the appearance of an intense band at approximately

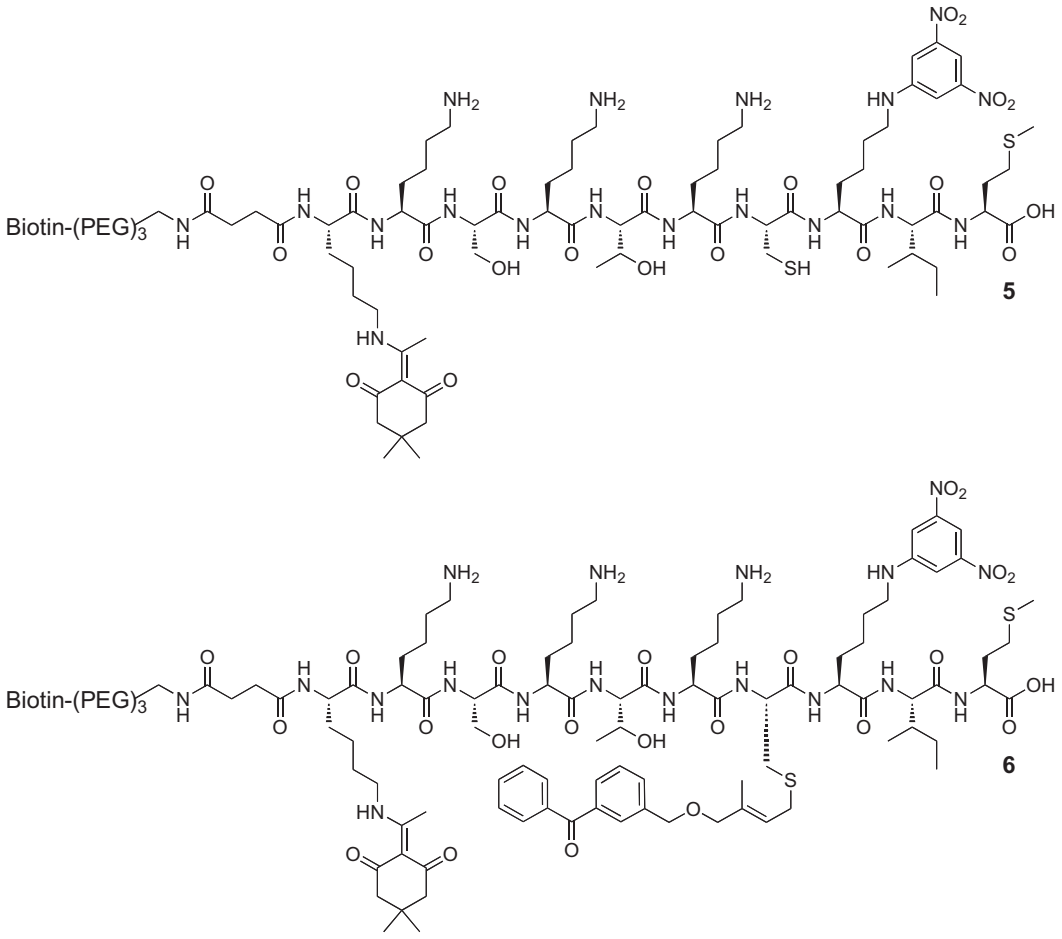

Figure 6. Structures of biotinylated peptides **5**, containing a free thiol, and **6** following alkylation using the benzophenone-modified isoprenoid, **3**.

Table 1
Kinetic parameters for substrate activity of Rce1p with benzophenone-modified photoprobe **4** and farnesylated peptide **2**.

Substrate	Kinetic parameters		
	K_M (μM)	V_{max} (nmol/min/mg protein)	V_{max}/K_M (min/mg protein)
2 ($n = 2$)	6.4 ± 0.2	5.6 ± 0.1	5.1×10^{-4}
4 ($n = 5$)	1.9 ± 0.3	0.093 ± 0.005	4.8×10^{-5}

40 kDa (Fig. 10, Lane 3), consistent with the predicted mass of HA-epitope tagged Rce1p (40.75 kDa). This data indicates that Rce1p can be effectively captured upon crosslinking to a biotinylated probe. Importantly, no HA-containing bands were detected in the photolysis reaction performed on membranes lacking Rce1p (Fig. 10, Lane 4). A small amount of Rce1p was observed in the absence of irradiation (Fig. 10, Lane 1) that was not present in the membranes lacking Rce1p (Fig. 10, Lane 2), indicating that the enzyme has some nonspecific affinity for the streptavidin beads used

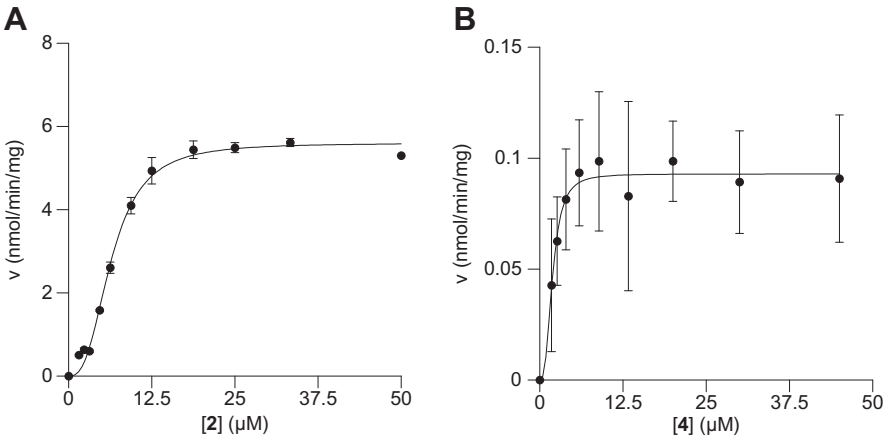

Figure 7. Kinetic analysis of Rce1p-catalyzed hydrolysis of farnesylated peptide, **2**, and benzophenone-modified substrate **4**. (A) Rate versus substrate concentration using peptide **2**. (B) Rate versus substrate concentration using peptide **4**.

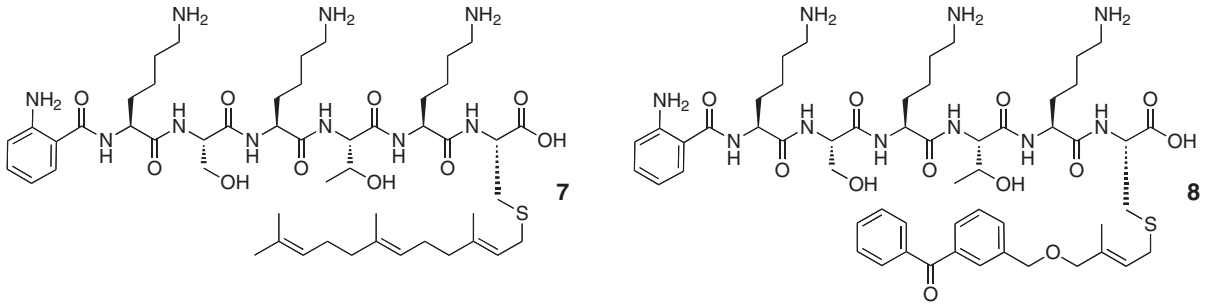

Figure 8. Structures of products **7** and **8** derived from Rce1p-catalyzed proteolysis of **2** and **4**, respectively.

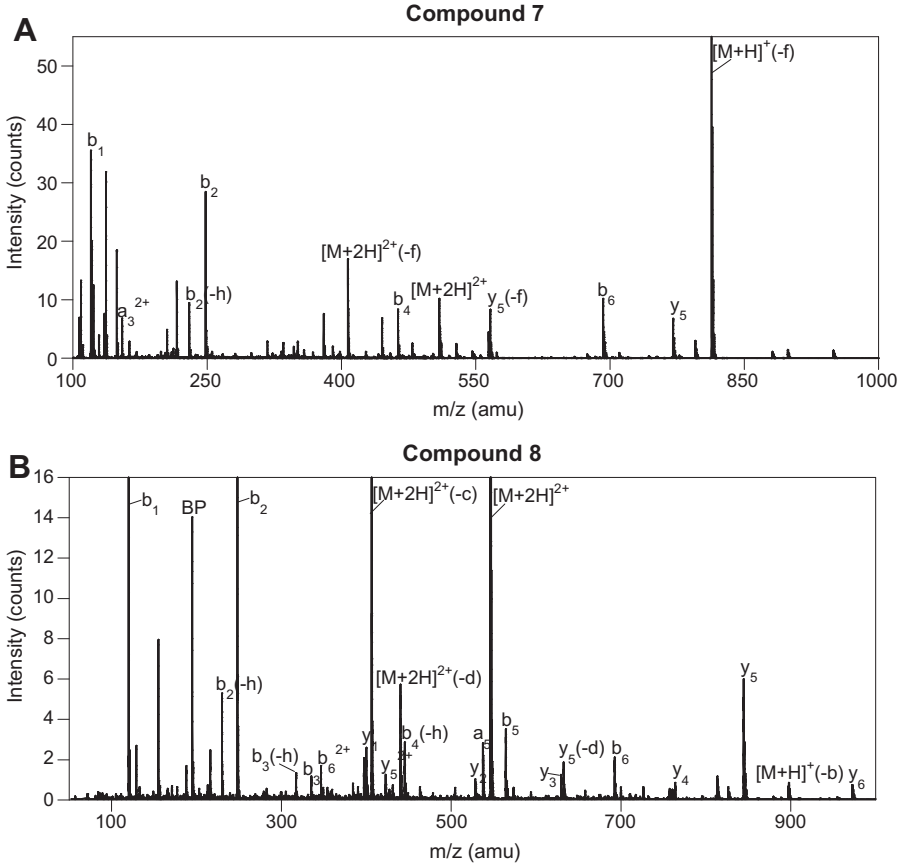

Figure 9. ESI-MS–MS of products obtained from Rce1p-catalyzed proteolysis of substrate peptides **2** and **4**. (A) Farnesylated proteolysis product **7** (-f, loss of farnesyl ($C_{15}H_{25}$); -h, loss of H_2O). (B) Benzophenone-modified proteolysis product **8** (BP, benzophenone fragment, (3-benzoylphenyl)methylium ($C_{14}H_{11}O^+$); -b, loss of benzophenone fragment; -c, loss of (*E*)-(3-(((2-methylbut-2-en-1-yl)oxy)methyl)phenyl)(phenyl)methanone ($C_{19}H_{20}O_2$); -d, loss of benzophenone–H_2O, (3-(hydroxymethyl)phenyl)(phenyl)methanone ($C_{14}H_{13}O_2$); -h, loss of H_2O).

in the pull-down process. That nonspecific binding was decreased by raising the detergent concentration in the wash step of the pull-down experiment from 0.1% SDS (data not shown) to 1.5% SDS (see Fig. 10) but could not be completely eliminated. It is unlikely, however, that the weak nature of nonspecific binding will interfere in subsequent efforts to identify the site of crosslinking.

2.5. Competition experiments using 2 and 6

Next, to examine the specificity of **6**, we studied the ability of the benzophenone-containing probe to label Rce1p upon photolysis in the presence of a competitor. Farnesylated peptide **2**, a known substrate for Rce1p, was selected as the competitor. Due to the limited solubility of **2**, the concentration of **6** employed was reduced to 0.75 μM. This allowed a larger excess of **2** to be used. Thus, irradiation of **6** in the presence of 50 μM **2** resulted in a significant reduction (60% of that observed in the absence of **2**) in crosslinked Rce1p (Fig. 11, Column 2). The extent of crosslinking by **6** was further reduced (to 31% of that observed in the absence of **2**) by increasing the concentration of **2** to 100 μM in the photolysis reaction (Fig. 11, Column 3). Overall, these results are consistent with the conclusion that probe **6** is labeling the active site of Rce1p.

3. Conclusions

Rce1p catalyzes the proteolytic removal of C-terminal tripeptides from prenylated proteins and is a possible target for the

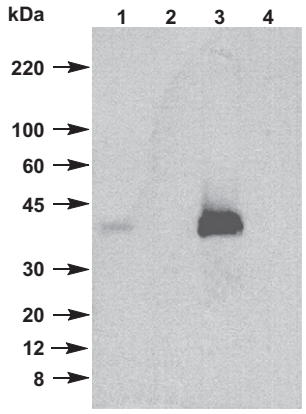

Figure 10. Western blot analysis of photolabeling of Rce1p with probe **6** detected with anti-HA following SA pull-down and SDS–PAGE separation. Lane 1: Rce1p-containing membranes (*RCE1*) and **6** (15 μM), no UV irradiation. Lane 2: membranes lacking Rce1p (*rce1*Δ) and **6** (15 μM), no UV irradiation. Lane 3: Rce1p-containing membranes (*RCE1*) and **6** (15 μM), with UV irradiation. Lane 4: membranes lacking Rce1p (*rce1*Δ) and **6** (15 μM), with UV irradiation. Results shown are representative of those obtained in three independent experiments.

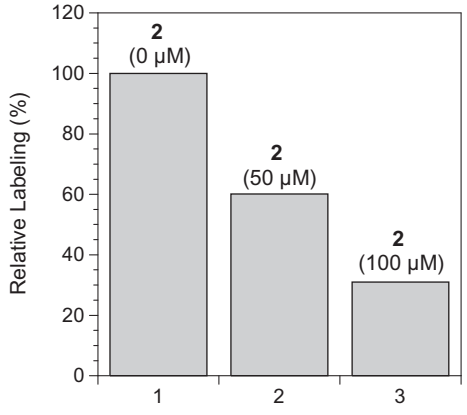

Figure 11. Densitometric quantification of western blot analysis of photolabeling of Rce1p with probe **6** in the presence of competitor **2** detected with anti-HA following SA pull-down and SDS–PAGE separation. Column 1: Rce1p-containing membranes (*RCE1*) and probe **6** (0.75 μM) with UV irradiation. Column 2: Rce1p-containing membranes (*RCE1*), probe **6** (0.75 μM), and farnesylated competitor **2** (50 μM) with UV irradiation. Column 3: Rce1p-containing membranes (*RCE1*), probe **6** (0.75 μM), and farnesylated competitor **2** (100 μM) with UV irradiation.

design of anti-cancer agents. Site-directed mutagenesis has been used to identify conserved residues that may be required for Rce1p catalytic activity.[1,10] Both competitive inhibitors and active site directed, irreversible affinity labeling reagents have been designed to characterize the structure and identify the mechanistic class of Rce1p.[11,32,33] Photoaffinity labeling reagents represent an additional means to evaluate this membrane-associated protease. The introduction of a photolabile moiety to the prenylcysteine of substrate peptides offers potential utility for structural analysis of Rce1p. The benzophenone-containing compounds are reasonable structural mimics of their farnesylated counterparts and their ability to crosslink to neighboring residues upon photoactivation makes them an appealing tool.

To gain further understanding into the structure of Rce1p, a peptide substrate containing a benzophenone-functionalized isoprenoid was designed, prepared, and tested to study its efficacy as a Rce1p substrate. Using a continuous spectrofluorometric assay it was shown that this substrate (**4**) is processed by Rce1p with an efficiency (V_{max}/K_M) that is approximately 18-fold lower than that for the related farnesylated peptide (**2**). However, with a K_M of 1.9 μM, **4** demonstrated 3.4-fold higher binding affinity than **2**. ESI-MS–MS was employed to confirm the site of proteolysis. It should be noted that the inclusion of the Abz fluorophore and Dnp quencher in **4** greatly simplified kinetic analysis and purification of the cleaved product for subsequent structural analysis. Photolysis of a biotinylated version of the probe (**6**) followed by streptavidin pull-down, SDS–PAGE and Western blot analysis demonstrated that Rce1p can be labeled by the probe and that the resulting crosslinked protein can be isolated. Photolysis in the presence of both probe **6** and a farnesylated peptide competitor **2** reduced the extent of labeling. Taken together, these results demonstrate that benzophenone-containing peptides can serve as bone fide substrates for Rce1p and suggest that the enzyme is being labeled in the active site. Efforts to use this probe to identify residues in the active site of Rce1p are ongoing.

4. Experimental

4.1. General

Analytical TLC was performed on precoated (0.25 mm) Silica Gel 60F-254 plates purchased from E. Merck. Flash chromatography silica gel (60–120 mesh) was obtained from E. M. Science. Fmoc-Cys(Me)-OH was from Bachem and Fmoc-Lys(Abz)-OH, Fmoc-Lys(Dnp)-OH, Fmoc-Lys(Dde)-OH were from Nova Biochem. Preloaded CLEAR-Acid resins were from Peptides International. TFP–PEG_3–biotin was from Thermo Scientific. All other reagents were from Sigma–Aldrich. Reactions were performed at room temperature unless otherwise noted. RP-HPLC analysis was carried out using a Beckman Model 125/168 instrument equipped with a diode array UV detector. Preparative RP-HPLC separations employed a Phenomenex Luna 10 μ 100 Å C_{18}(2) column (10.00 mm × 25 cm). Analytical RP-HPLC separations employed a Varian Microsorb-MV 5 μ 100 Å C_{18} column (4.6 mm × 25 cm) with a Phenomenex Luna 5 μ guard cartridge (4.6 mm × 3 cm). Retention times (t_R) were based on analytical RP-HPLC using a linear gradient from 100% H_2O to 40% H_2O/60% CH_3CN over 60 min and a flow rate of 1.0 mL/min. Peptide concentrations were determined by UV spectroscopy of the Dnp quencher (349 nm, ε = 18,000 cm^{-1} M^{-1}). The fluorogenic peptides were protected from light as much as possible to avoid bleaching of the Abz fluorophore. C_{18} Sep-Pak columns were purchased from Waters. Protein precipitation kit was purchased from CalBiochem. Spin columns, streptavidin resin, and ECL substrate solutions were purchased from Pierce. Autoradiographic film was purchased from Bioexpress. Antibodies were purchased from Covance and GE Healthcare.

4.1.1. Yeast strains and plasmids

The parent yeast strain used in this study is SM3614 (*MAT*a *trp1 leu2 ura3 his4 ste24::LEU2rce1::TRP1*).[34] SM3614 was transformed with plasmid pRS316 (*CEN URA3*) or pWS479 (*2μURA3*P_{PGK}*-RCE1::-HA*) to create *rce1*Δ and wildtype (WT) strains, respectively.[10,35] Yeast transformations were carried out according to established methods.[36] Transformed yeast strains were grown at 30 °C in synthetic complete medium lacking uracil (SC-Ura).

4.2. Chemical synthesis

4.2.1. Synthesis of Abz-KSKTKCK(Dnp)IM, 1

The linear peptide sequence was synthesized by Fmoc-based SPPS on either an Applied Biosystems 433A or a Pioneer automated peptide synthesizer using CLEAR-Acid resin. It was then cleaved from the resin using 4.0 mL of freshly-prepared, Reagent K[37] (TFA/water/thioanisole/phenol/ethanedithiol 82.5:5:5:5:2.5) for 2.5 h, precipitated with 100.0 mL of anhydrous ether, and centrifuged to

form a pellet. The pellet was washed with ether, solubilized in a small amount of DMF, diluted in 0.1% aqueous TFA, and filtered by vacuum filtration. The crude material was purified by RP-HPLC using 0.1% TFA/H_2O (solvent A) and 0.1% TFA/CH_3CN (solvent B) with the following elution profile performed at 5.0 mL/min: 5.0 min 0% solvent B; linear gradient from 0% to 60% solvent B over 120 min. The solvent mixture was removed by lyophilization to afford 20.0 mg (30%) of **1**. Purity by RP-HPLC: 94.4%, t_R = 41 min., ESI-MS (*m*/*z*): $[M+H]^+$ calcd for $C_{58}H_{94}N_{16}O_{17}S_2$ 1351.7236, found 1351.4039; $[M+2H]^{2+}$ calcd for $C_{58}H_{95}N_{16}O_{17}S_2$ 676.3212, found 676.3596; $[M+3H]^{3+}$ calcd for $C_{58}H_{96}N_{16}O_{17}S_2$ 451.2141, found 451.2424.

4.2.2. Synthesis of Abz-KSKTKC(C5BP)K(Dnp)IM, 4

Peptide **1** (10.0 mg, 7.0 μmol, 1.0 equiv) was dissolved in 10.0 mL of 2:1:1 DMF/*n*-butanol/0.1% aqueous TFA (v/v/v) and (*E*)-4-(3′-benzoylbenzyloxy)-3-methyl-2-buten-1-bromide, **3** (10.0 mg, 30 μmol, 4.0 equiv), synthesized according to published methods,[15,19] was then added. $Zn(OAc)_2$ (8.0 mg, 40 μmol, 5.0 equiv) was dissolved in a small amount 0.1% aqueous TFA and added to the reaction. The reaction was monitored by RP-HPLC and was complete in 2 h. It was then diluted with 0.1% aqueous TFA before RP-HPLC purification. The crude material was purified by RP-HPLC using 0.1% TFA/H_2O (solvent A) and 0.1% TFA/CH_3CN (solvent B) and the following elution profile performed at 5.0 mL/min: 5.0 min 0% solvent B; linear gradient from 0% to 60% solvent B over 120 min. The solvent mixture was removed by lyophilization yielding 3.3 mg (27%) of **4**. Purity by RP-HPLC: 91.5%, t_R = 60 min., ESI-MS (*m*/*z*): $[M+2H]^{2+}$ calcd for $C_{77}H_{113}N_{16}O_{19}S_2$ 815.3866, found 815.3873; $[M+3H]^{3+}$ calcd for $C_{77}H_{114}N_{16}O_{19}S_2$ 543.9244, found 543.9283. UV ε_{349} = 18,000 mM^{-1} cm^{-1}.

4.2.3. Synthesis of biotin-K(Dde)KSKTKCK(Dnp)IM, 5

The linear peptide sequence was synthesized by Fmoc-based SPPS on a peptide synthesizer using CLEAR-Acid resin. The final Fmoc protecting group was selectively removed from the protected peptide resin (121.0 mg, 40 μmol, 1.0 equiv) by tumbling it 15 min in a 1:4 (v/v) mixture of piperidine in DMF (5.0 mL), followed by washing with DMF (2 × 5.0 mL) and methylene chloride (1 × 5.0 mL). TFP–PEG_3–Biotin (100 mg, 140 μmol, 4.0 equiv dissolved in 1.0 mL DMF) was added and it was coupled to the N-terminus using DIEA (49.0 μL, 300 μmol, 8.0 equiv) and tumbling 48 h. The resin was then washed with DMF (2 × 5.0 mL) and methylene chloride (4 × 5.0 mL) and dried in vacuo. The peptide was cleaved from the resin using 4.0 mL of freshly-prepared, Reagent K (TFA/water/thioanisole/phenol/ethanedithiol 82.5:5:5:5:2.5) for 2.5 h, precipitated with anhydrous ether (100.0 mL), and centrifuged to form a pellet. The pellet was washed with ether, solubilized in a small amount of DMF, diluted in 0.1% aqueous TFA, and filtered by vacuum filtration. The crude product was purified by RP-HPLC using 0.1% TFA/H_2O (solvent A) and 0.1% TFA/CH_3CN (solvent B) with the following elution profile performed at 5.0 mL/min: 5.0 min 0% solvent B; linear gradient from 0% to 60% solvent B over 120 min. The solvent mixture was removed by lyophilization to afford 20 mg (28%) of **5**. Purity by RP-HPLC: 75.6%, t_R = 52 min., ESI-MS (*m*/*z*): $[M+2Na]^{2+}$ calcd for $C_{91}H_{153}N_{21}O_{26}S_3Na_2$ 1049.0127, found 1048.6907; $[M+2Na+H]^{3+}$ calcd for $C_{91}H_{154}N_{21}O_{26}S_3Na_2$ 699.6751, found 699.4679; $[M+2Na+2H]^{4+}$ calcd for $C_{91}H_{155}N_{21}O_{26}S_3Na_2$ 525.0063, found 524.5923.

4.2.4. Synthesis of biotin-K(Dde)KSKTKC(C5BP)K(Dnp)IM, 6

Peptide **5** (15.0 mg, 7.0 μmol, 1.0 equiv) was dissolved in 2.0 mL of 2:1:1 DMF/*n*-butanol/0.1% aqueous TFA (v/v/v) and (*E*)-4-(3′-benzoylbenzyloxy)-3-methyl-2-buten-1-bromide, **3** (10.5 mg, 30 μmol, 4.0 equiv), was added. $Zn(OAc)_2$ (8.0 mg, 40 μmol, 5.0 equiv) was dissolved in a small amount 0.1% aqueous TFA and added to the reaction. The reaction was monitored by RP-HPLC. Following 2 h of reaction time, 200 μL of BME was added (1.43 M) to quench the reaction and reverse any sulfonium salt formation.[29] It was stirred in BME 2.5 h, filtered, and diluted with 0.1% aqueous TFA before RP-HPLC purification. The crude was purified by RP-HPLC using 0.1% TFA/H_2O (solvent A) and 0.1% TFA/CH_3CN (solvent B) and the following elution profile performed at 2.5 mL/min: 5.0 min 0% solvent B; linear gradient from 30% to 100% solvent B over 140 min. The solvent mixture was removed by lyophilization to afford 12 mg (74%) of **6**. Purity by RP-HPLC: 97.0%, t_R = 61 min., ESI-MS (*m*/*z*): $[M+2Na]^{2+}$ calcd for $C_{110}H_{171}N_{21}O_{28}S_3Na_2$ 1188.0780, found 1187.8760; $[M+2Na+H]^{3+}$ calcd for $C_{110}H_{172}N_{21}O_{28}S_3Na_2$ 792.3853, found 792.2635; $[M+2Na+2H]^{4+}$ calcd for $C_{110}H_{173}N_{21}O_{28}S_3Na_2$ 594.5390, found 594.4493. UV ε_{349} = 18,000 mM^{-1} cm^{-1}.

4.3. Kinetic analysis

4.3.1. In vitro fluorescence-based Ca_1a_2X proteolysis assay

Carbonate-washed membrane fractions containing yeast Rce1p were used as the source of enzyme. Membranes were isolated according to reported methods and prepared as 1.0 mg/mL total protein stock solutions in lysis buffer (50 mM Tris, pH 7.5, 0.2 M sorbitol, 1 mM EDTA, 0.2% NaN_3) containing a protease inhibitor cocktail (1 μg/mL each chymostatin, leupeptin, pepstatin; 0.85 μg/mL aprotinin; 1 mM PMSF) and stored at −80 °C.[8,30] The membranes were diluted with an equal volume of assay buffer (100 mM Hepes, pH 7.5, 5 mM $MgCl_2$) to 0.5 mg/mL and preincubated in a metallic block for 10 min at 30 °C. Substrates **2** and **4** were serially diluted in assay buffer from their stock concentrations (100 and 90 μM, respectively) into a 96-well V-shaped microtiter plate (three 15 μL replicates) and transferred to 384-well black flat bottom microtiter plate; 10 concentration points for each substrate. The plate was covered with a lid and incubated 10 min at 30 °C. Assays were initiated with the addition of 15 μL of diluted membranes to the diluted substrates in the wells, resulting in a final membrane protein concentration of 0.25 mg/mL, substrate concentrations ranging 0–50 μM (**2**), or 0–45 μM (**4**), and a total assay volume of 30 μL. The fluorescence in the samples was measured every 30–60 s for 1 h at 30 °C with 5 s shaking before each reading at 340/420 nm excitation/emission wavelengths on a BioTek Synergy™ HT microplate fluorometer.

4.3.2. Kinetic analysis

Data from experiments was collected as replicates (two for **2** and five for **4**) and analyzed using GraphPad Prism 4.0. Data was fit to the four parameter Hill Equation $Y = V_{max} * X^h/(K_M^h + X^h)$ without constraints after providing approximate initial values. Prior to analysis, the data (RFU_{sample}) was corrected to take into account the observation that RFU and product formation are proportional but do not have a 1:1 relationship. In part, this is due to intermolecular quenching effects that increase as substrate concentration increases. A standard curve was created using trypsin to determine the maximum fluorescence obtainable at various substrate concentrations ($RFU_{trypsin}$). Both **2** and **4** contain trypsin cleavage sites. A graph of $RFU_{trypsin}$ versus substrate concentration was plotted for each substrate and a best-fit line determined (**2**, $Y = 116.29X$, $R^2 = 0.993$; **4**, $Y = 168.23X$, $R^2 = 0.9834$). The equations of the curves were used to extract the actual product concentration for partially reacted samples (RFU_{sample}). These values were used for Prism analysis.

4.4. Mass spectrometric analysis

4.4.1. Large-scale proteolysis reactions for product structure identification

Stock concentrations of **2** and **4** were diluted in Hepes buffer (100 mM Hepes, 5 mM $MgCl_2$, pH 7.5) to 150 μM. Rce1p-contain-

ing membranes were then added to a final concentration of 0.3 mg/mL (total protein). The samples were mixed briefly and incubated in a 30 °C water bath for 3–3.5 h. The crude product was purified using Sep-Pak reversed phase C_{18} columns equilibrated in solvent A (0.1% TFA/H_2O). The reaction mixtures containing proteolyzed peptide were applied to the columns and fractions were eluted with a stepwise gradient from 0% to 100% B using 4–6 mL each mixture of solvents A and B. The individual fractions were evaluated by ESI-MS, which showed the doubly-charged parent ions of the products of cleavage in the 30%, 40%, and 50% B fractions. These fractions were lyophilized separately and re-dissolved in solvent A before MS analysis.

4.4.2. MS–MS analysis of farnesylated and benzophenone-modified substrates following enzymatic processing by Rce1p

The lyophilized samples from proteolysis of **2** and **4** were dissolved in 10 μL of 0.1% aqueous TFA and further diluted 2:50 in 0.1% FA/CH_3CN prior to MS analysis. MS was performed using 10 μL injections (CI 25-32) and 5 min. data acquisition on a QSTAR Pulsar *i* quadrupole-TOF (time-of-flight) mass spectrometer equipped with a turbo ionspray source. The doubly-charged $[M+2H]^{2+}$ species (m/z = 509.3 and m/z = 546.4, respectively) for the predicted products **7** and **8** were most abundant in the 40% B fractions and were selected for subsequent MS–MS analysis. MS results were evaluated using Analyst software.

4.5. Photoaffinity labeling

All photolysis reactions were conducted at 4 °C in a UV Rayonet photoreactor (Model # RPR-100, Southern New England Ultraviolet Co.) equipped with sixteen RPR-2537 Å lamps and a circulating platform that allows up to 13 samples to be irradiated simultaneously. All reactions (135 μL) were performed in silanized quartz test tubes (10 × 43 mm) and contained 100 mM Hepes, 5 mM $MgCl_2$, pH 7.5, 15 μM **6**, and 0.2 μg/μL membranes containing or lacking Rce1p. The reactions were photolyzed for 30 min using the apparatus described above and quenched by flash-freezing in N_2 (l).

4.5.1. Pull-down of photolabeled Rce1p on streptavidin beads followed by Western blot analysis

Irradiated samples were thawed and concentrated using protein precipitation. The concentrated samples were solubilized in RIPA buffer [150 μL; 150 mM NaCl, 50 mM Tris·HCl, 1.0% (w/v) sodium deoxycholate, 1.0% (v/v) Triton X-100] containing 1.5% (w/v) SDS and added to spin columns containing streptavidin agarose resin (0.1–0.5 μg of protein/μL resin pre-equilibrated with RIPA/1.5% SDS) and incubated 1–1.5 h at room temperature. Following brief centrifugation, the resin was washed five times with RIPA/1.5% SDS.

The samples were eluted by adding 2× sample buffer [15 μL; 4.0% SDS, 20% glycerol (v/v), 125 mM Tris·HCl (pH 6.8), 10% 2-mercaptoethanol (v/v), 0.004% bromophenol blue (w/v)] to each spin column, heating to 95 °C for 5 min followed by centrifugation, and repeated for a total of 30 μL of sample. The samples were then combined with an additional 30 μL of 2× sample buffer before SDS–PAGE (12% Tris–glycine gels) electrophoresis, and transfer to PVDF membranes.

The PVDF membranes were blocked with 5% (w/v) casein in TBST [25 mM Tris, 150 mM NaCl, 0.1% Tween-20 (v/v), pH 7.4] for 1 h, then incubated in 2.5% (w/v) casein in TBST containing anti-HA antibody [mAb HA.11 (HA, 16B12, flu tag), 1:10,000 (Covance)] overnight at 4 °C. The membranes were then washed with TBST and PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4] and incubated with 2.5% (w/v) casein in TBST containing secondary antibody [ECL anti-mouse IgG, horseradish peroxidase-linked (NA931, from sheep), 1:10,000 (GE Healthcare)] for 2 h at room temperature. Membranes were washed and visualized using enhanced chemiluminescence (ECL) on autoradiographic film.

4.5.2. Competition experiments using 2 and 6

Photolysis reactions were conducted as described in Section 4.5. All reactions (200 μL) contained 100 mM Hepes, 5 mM $MgCl_2$, pH 7.5, 0.75 μM **6**, and 0.25 μg/μL membranes containing Rce1p. Compound **2** was used at 50 μM and 100 μM in the assays evaluating competition. Pull-down and Western blotting were performed as described in section 4.5.1.

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Supplementary data

Supplementary data (RP-HPLC chromatograms for **1**, **4**, **5**, and **6** and summary tables of mass spectral data for **2**, **4**, **7**, and **8**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.024.

References and notes

1. Dolence, J. M.; Steward, L. E.; Dolence, E. K.; Wong, D. H.; Poulter, C. D. *Biochemistry* **2000**, *39*, 4096.
2. Quellhorst, G. J.; Allen, C. M.; Wessling-Resnick, M. *J. Biol. Chem.* **2001**, *276*, 40727.
3. Kim, E.; Ambroziak, P.; Otto, J. C.; Taylor, B.; Ashby, M.; Shannon, K.; Casey, P. J.; Young, S. G. *J. Biol. Chem.* **1999**, *274*, 8383.
4. Dolence, E. K.; Dolence, J. M.; Poulter, C. D. *Bioconjugate Chem.* **2001**, *12*, 35.
5. Otto, J. C.; Kim, E.; Young, S. G.; Casey, P. J. *J. Biol. Chem.* **1999**, *274*, 8379.
6. Brown, M. J.; Milano, P. D.; Lever, D. C.; Epstein, W. W.; Poulter, C. D. *J. Am. Chem. Soc.* **1991**, *113*, 3176.
7. Dorman, G.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661.
8. Porter, S. B.; Hildebrandt, E. R.; Breevoort, S. R.; Mokry, D. Z.; Dore, T. M.; Schmidt, W. K. *Biochim. Biophys. Acta* **2007**, *1773*, 853.
9. Hollander, I.; Frommer, E.; Aulabaugh, A.; Mallon, R. *Biochim. Biophys. Acta* **2003**, *1649*, 24.
10. Plummer, L. J.; Hildebrandt, E. R.; Porter, S. B.; Rogers, V. A.; McCracken, J.; Schmidt, W. K. *J. Biol. Chem.* **2006**, *281*, 4596.
11. Ma, Y. T.; Gilbert, B. A.; Rando, R. R. *Biochemistry* **1993**, *32*, 2386.
12. Pei, J.; Grishin, N. V. *Trends Biochem. Sci.* **2001**, *26*, 275.
13. Hollander, I.; Frommer, E.; Mallon, R. *Anal. Biochem.* **2000**, *286*, 129.
14. Kale, T. A.; Turek, T. C.; Chang, V.; Gautam, N.; Distefano, M. D. *Methods Enzymol.* **2002**, *344*, 245.
15. Kale, T. A.; Raab, C.; Yu, N.; Dean, D. C.; Distefano, M. D. *J. Am. Chem. Soc.* **2001**, *123*, 4373.
16. Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483.
17. Rajagopalan, K.; Chavan, A. J.; Haley, B. E.; Watt, D. S. *J. Biol. Chem.* **1993**, *268*, 14230.
18. Kale, T. A.; Raab, C.; Yu, N.; Aquino, E.; Dean, D. C.; Distefano, M. D. *J. Labelled Compd. Radiopharm.* **2003**, *46*, 29.
19. Turek, T. C.; Gaon, I.; Distefano, M. D.; Strickland, C. L. *J. Org. Chem.* **2001**, *66*, 3253.
20. Kale, T. A.; Distefano, M. D. *Org. Lett.* **2003**, *5*, 609.
21. Hovlid, M. L.; Edelstein, R. L.; Henry, O.; Ochocki, J.; DeGraw, A.; Lenevich, S.; Talbot, T.; Young, V. G.; Hruza, A. W.; Lopez-Gallego, F.; Labello, N. P.; Strickland, C. L.; Schmidt-Dannert, C.; Distefano, M. D. *Chem. Biol. Drug Des.* **2010**, *75*, 51.
22. Baba, T.; Allen, C. M. *Biochemistry* **1984**, *23*, 1312.
23. Yokoyama, K.; McGeady, P.; Gelb, M. H. *Biochemistry* **1995**, *34*, 1344.
24. Jitkova, J.; Carrigan, C. N.; Poulter, C. D.; Krylov, S. N. *Anal. Chim. Acta* **2004**, *521*, 1.
25. Edelstein, R. L.; Distefano, M. D. *Biochem. Biophys. Res. Commun.* **1997**, *235*, 377.
26. 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.
27. Alexander, M.; Gerauer, M.; Pechlivanis, M.; Popkirova, B.; Dvorsky, R.; Brunsveld, L.; Waldmann, H.; Kuhlmann, J. *ChemBioChem* **2009**, *10*, 98.
28. Kuhlmann, J.; Tebbe, A.; Volkert, M.; Wagner, M.; Uwai, K.; Waldmann, H. *Angew. Chem., Int. Ed.* **2002**, *41*, 2546.
29. Xue, C. B.; Becker, J. M.; Naider, F. *Tetrahedron Lett.* **1992**, *33*, 1435.

30. Manandhar, S.; Hildebrandt, E. R.; Schmidt, W. K. *J. Biomol. Screen.* **2007**, *12*, 983.
31. DeGraw, A. J.; Zhao, Z.; Strickland, C. L.; Taban, A. H.; Hsieh, J.; Jeffries, M.; Xie, W.; Shintani, D. K.; McMahan, C. M.; Cornish, K.; Distefano, M. D. *J. Org. Chem.* **2007**, *72*, 4587.
32. Roberts, M. J.; Troutman, J. M.; Chehade, K. A. H.; Cha, H. C.; Kao, J. P. Y.; Huang, X.; Zhan, C.; Peterson, Y. K.; Subramanian, T.; Kamalakkannan, S.; Andres, D. A.; Spielmann, H. P. *Biochemistry* **2006**, *45*, 15862.
33. Chen, Y.; Ma, Y.; Rando, R. R. *Biochemistry* **1996**, *35*, 3227.
34. Tam, A.; Nouvet, F. J.; Fujimura-Kamada, K.; Slunt, H.; Sisodia, S. S.; Michaelis, S. *J. Cell Biol.* **1998**, *142*, 635.
35. Sikorski, R. S.; Hieter, P. *Genetics* **1989**, *122*, 19.
36. Elble, R. *Biotechniques* **1992**, *13*, 18.
37. King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255.