Synthesis of Peptide-Based Small Molecule Probes to Study Redox Status and Protein Prenylation

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Dedication

The first drafts of this are dedicated to Grandma Elmyra Anna Emilie Sabatke Kage, on what would have been her 98th birthday. With love, forever and always.

And also, to my William- for believing in me.

Abstract

The use of solid phase peptide synthesis to produce molecular probes for chemical biology applications is an efficient and tunable process allowing for the synthesis of tools for a wide range of applications. The modular nature of peptide synthesis makes it relatively simple to change features of a probe to suit whatever needs are necessary. The sidechains of the amino acid building blocks serve as convenient handles for the addition of fluorophores or other tags.

In this thesis, peptides are used as building blocks to make a redox probe and several analogues of an internally quenched Ste24p cleavage probe. I also examine the consequences of solid phase synthesis, specifically racemization, when cysteine is the C-terminal residue. The synthesis of the redox probe was modified to improve the ease of its production. The internally quenched Ste24p cleavage probe was applied to show that C-terminal modifications of the probe do not appear to influence the cleavage kinetics of the Ste24p protease.

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Chapter 1: Advances in the efficient synthesis of a Peptide-Based Redox Probe for Biological Redox State Analysis.

1.1 Abstract

Changes in the redox environment in biological systems have significant implications for disease. For example, altered mitochondrial matrix redox environments are associated with disease states such as cancer, diabetes and Alzheimer's, as well as cell death mechanisms.[1-2] A high-throughput method to quantify biological redox status across biological fluids, cells from culture and even tissues would mark an important advance in how we probe biological redox chemistry. These redox-sensitive sensors could allow for the quantification of changes in the redox environment of cells, and potentially organelles, as a function of disease. The probes could be applied to biological fluids (blood or cerebral spinal fluid) as well as cytosolic measurements. In this work, the synthesis of previously described small molecule probes is modified to improve ease of production.[3-6]

1.2 Background

The proposed probes are designed to equilibrate with the ratio of glutathione (GSH) to glutathione disulfide (GSSG) in their environment by thiol disulfide exchange between cysteine residues in the sensor's peptide backbone.[1] In this manner, the peptides operate as Forster Resonance Energy Transfer (FRET) switches. When the adjacent cysteine residues are linked by a disulfide, the resulting cyclization of the peptide holds the incorporated donor/acceptor apart, preventing energy transfer and decreasing emission at the FRET wavelength. The ratio of donor to acceptor fluorescence is a measure of the ratio

of oxidized to reduced peptide, which is a measure of the ratio of GSH/GSSG in the mitochondrial matrix. This ratio can be converted to a numerical redox potential of the mitochondrial matrix with the Nernst equation. With the use of spectrofluorimeters and related instrumentation, fluorescence ratios and in turn the redox poise in biological fluid, cells, and potentially organelles, can be quantified.

The redox environment of a cellular compartment is defined as the summation of the products of the reduction potential and the concentration of the reduced species of each chemical redox couple in the compartment.[2] Because it is difficult to identify and quantify every redox player in a compartment, the redox state of a redox buffer in the compartment to be studied can be used as an indicator of the redox environment. For example, glutathione is the primary redox buffer in biological systems. In the presence of oxidizers, two molecules of glutathione undergo the reversible formation of a disulfide bond to make GSSG. The ratio of GSH/GSSG is a measure of the redox state of the glutathione buffer system and an indicator of the redox environment.

Currently, making measurements of redox status is constrained by several limitations. The most sensitive methods rely on direct quantification of oxidized and reduced glutathione by liquid chromatography.[1-2] These methods require intensive sample preparation during which artifacts in redox status can occur. They are not high-throughput by any means, and due to necessity of complex instrumentation and multi-step sample preparation they preclude point-of care or single cell measurements. Other, higher throughput methods in the form of plate-reader based ELISA assays are often employed to as faster options, but these lack the sensitivity of the LC analyses and do nothing to

overcome the inability to measure single cell redox status, which is a critical feature if we are to be able to study redox status in the context of cellular heterogeneity.[1-2]

With these known limitations in mind, we envisioned a synthetic peptide-based probe that would improve upon the current techniques in several ways. First, the probe would be applicable for use in a variety of widely available applications and instrumental set-ups including plate readers and fluorimeters. Second, the structure would be easily tunable to a variety of reduction potential midpoints and an array of fluorimeter and plate reader excitation and emission wavelengths. Third, the probe would be applicable for both real-time and reversible measurements. Lastly, the possibility would exist to attach targeting moieties for intracellular and potentially intra-organelle measurements. The probe was based on an already published design and went through multiple variations on structure and methods.[3-5] We modified a synthetic route that promised to be relatively applicable to scale up, and we added some supporting information to a previously published route to the on-resin synthesis of C-terminally functionalized peptides.

1.3 Rationale

The probe structure (19) was originally developed by Lee et. Al in a publication from 2004, in which several structures were identified by computational analysis that would behave as a redox environment responsive molecular switch.[3] The structures are characterized by a four-amino acid backbone with a sequence engineered to form a beta-turn, flanked on either side by cysteine residues. The beta-turn encourages the formation of an intramolecular disulfide upon introduction to an oxidizing environment. The C-terminal cysteine is functionalized at its carboxylic acid with a carbostyril 124 flourophore

that serves as an internal standard and eliminates the need for a second entity for quantification purposes. It also acts as an emission sensitizer for the lanthanide (in this case, terbium) that is held at the N-terminal end of the probe in a chelator. This results in probe luminescence only when the disulfide bond is formed and the probe is in its cyclized state.

The readout the of probe is ratiometric, and measures the ratio of the emission 425 nm (carbostyril) /560 nm (Tb^{3+}). The emission intensity of the carbostyril 124 changes very little upon oxidation or reduction of the probe. However, in the oxidized (cyclized) state, the carbostyril is brought near the chelated terbium and functions as a sensitizer, enabling the characteristic emission of the chelated lanthanide.[3]

The probe works by equilibrating with the local ratio of oxidized to reduced glutathione. At any given redox potential, some of the pool will be oxidized (in the form of glutathione disulfide or GSSG) and some will be reduced (in the form of free glutathione, or GSH). The ratio of oxidized to reduced (or cyclized to linear) probe changes to match the ratio of GSSG:GSH. The ratio of the two colors of fluorescence can then be related to the ratio of oxidized to reduced glutathione. Equipped with that ratio of emission intensities and the Nernst equation, we can obtain a potential measurement for the environment of interest.

1.3.1 Redox Environment and Redox State: Definition and Quantification

The redox environment of a subcellular compartment is defined as the summation of the products of the reduction potential and concentration of the reduced species of each related redox couple in a given space. Mathematically, this is defined by Equation 1:

(1) $\text{redox environment} = \sum_{i=1}^{n\,(couple)} E_i \times [reduced\ species]_i$

The redox couples involved in this calculation are any biologically relevant redox couples, such as GSH/GSSG, NAD+/NADH (nicotinamide adenine dinucleotide), or NADP+/NADPH (nicotinamide adenine dinucleotide phosphate). To gain a comprehensive knowledge of the true redox environment as it is defined here, it is necessary to determine specific concentrations of every redox couple in the environment. Measurements of this nature are not possible when all redox couple identities and concentrations are not known. It is important to develop an appropriate way to estimate the redox environment and to define a system by which changes in the redox environment can be accurately quantified.

Because of glutathione's status as the primary cellular redox buffer it can be used as a redox environment indicator. Glutathione's concentration within the cell varies from compartment to compartment. In the cytosol, [GSH] has been found to be anywhere from 1 to 11mM. The nucleus has been shown to hold between 5 and 10% of the glutathione in an entire cell, at concentrations similar to those found in the cytosol. In the mitochondrial matrix, the total concentration of glutathione ranges from 5 to 11mM.[2]

Glutathione acts as a redox buffer in much the same way as an acid-base buffer, and changes in the redox state of this buffer system are widely accepted to indicate changes in redox environment. It reacts with hydrogen peroxide via glutathione peroxidase

(Equation 2) to form GSSG, which can then be reduced back to 2GSH by glutathione reductase and NADPH (Equation 3).

(2) $2GSH + H_2O_2 \xrightarrow[Peroxidase]{Glutathione} GSSG + 2H_2O$

(3) $GSSG + 2NADPH \xrightarrow[Reductase]{Glutathion} 2GSH + NADP^+$

In mitochondria, a ratio of greater than or equal to 100:1 [GSH]:[GSSG] is maintained enzymatically in the presence of oxidizing agents. The glutathione half-cell reaction is shown in Equation 4. It is a two-electron process.

(4) $GSSG + 2H^+ + 2e^- \rightarrow 2GSH$

To determine the concentration dependent redox potential of this half-cell, the Nernst equation, shown in Equation 5, is used.

(5) $\Delta E = \Delta E° - \frac{RT}{nF} lnQ$

In this equation, R is defined as the gas constant (R = 8.314 $JK^{-1}mol^{-1}$), T is the temperature in Kelvin, F is the Faraday constant (F = 9.6485 x 10^4 C mol^{-1}), *n* is the number of electrons being transferred, and $\Delta E°$ is defined as the standard half-cell reduction potential of the system. The "$\frac{RT}{nF} lnQ$" term can be simplified to "$\frac{59.1mV}{n} logQ$" when a conversion factor for the natural log term to $\log_{10}$, 2.303 is applied. Determination of "Q" for the reduction of GSSG is expressed in Equations 6 and 7.

(6) $Q = \frac{[product]}{[reactant]}$

(7) $Q = \frac{[GSH]^2}{[GSSG]}$

The standard half-cell potential of glutathione at pH=10 is +180mV. To correct for the more acidic pH (~7.8) in the mitochondrial matrix, the following equation is used.

(8) $\Delta E_{pH7.8} = \Delta E° - \frac{59.1}{n} log \frac{1}{[H^+]^2}$

Using Equation 6, where $\Delta E° = 180mV$, n=2 and $[H^+]$ = 1.58x10^{-8}, a half-cell potential for the glutathione in the mitochondrial matrix of -280mV is obtained. Where $\Delta E°$ is now -280mV, the expected ratio of [GSH]:[GSSG] can be determined using Equation 9.

(9) $\Delta E = -280mV - \frac{59.1}{2} log \frac{[GSH]^2}{[GSSG]}$

At -280mV and a total concentration of 10mM glutathione, the expected ratio of [GSH]:[GSSG] is 1:3. The total concentration of glutathione is represented by Equation 10.

(10) Total Glutathione = GSH + 2GSSG

It is important to note that the total concentration of glutathione in a compartment affects the final half-cell potential. This is due to the squaring of the concentration of GSH in the Q term, shown in Equation 7. Using Equation 8 where at pH 7.8, a ratio of 3:1 [GSH]:[GSSG] at a total concentration of 10mM gives a half-cell potential of -329mV. If the total concentration of glutathione doubled to 20mM while the ratio of reduced to oxidized glutathione held steady at 3:1, the half-cell potential decreases to -338mV. For

this reason, it is important to estimate the total concentration of glutathione when using the ratio of GSH/GSSG to determine the redox potential of the matrix environment.

	Reporter 1		Backbone		Reporter 2	
	Solid Phase?	Synthesized?	Solid Phase?	Synthesized?	Solid Phase?	Synthesized?
Method 1	yes	no	yes	yes	yes	no
Method 2	yes	yes	yes	yes	yes	yes
Published	yes	yes	yes	yes	no	yes

Figure 1:Comparison of methods for the synthesis of the proposed redox probe. In Method 1, no additional synthesis is required beyond that for the peptide backbone segment, which is done on solid phase. In Method 2, we need to synthesize both reporters which adds complexity to the synthesis, but both reporters can be incorporated on resin, which is still better than our alternative. In the previously published method, both reporters need to be synthesized and only one of them, reporter 1, can be incorporated using on-resin methodology.[**7**] This introduces significant complexity to the synthesis of this molecule. In the above figure, a green panel indicates a desired feature, while the orange indicates a less desired feature.

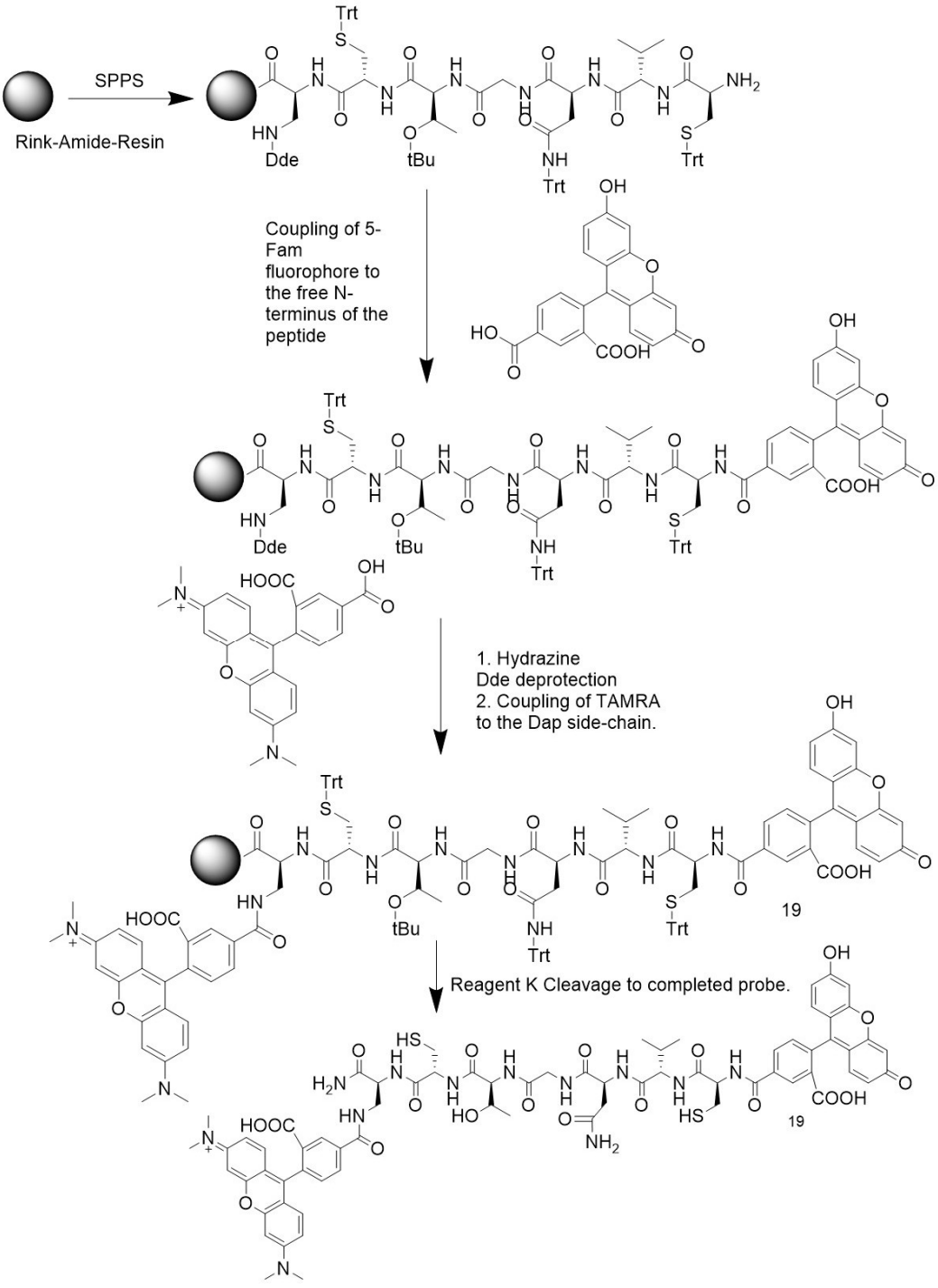
1.4 Methods: Fluorescent Probes

1.4.1 Synthesis of Fluorescently Modified Redox Probes: Method 1, Figure 1

Even though the publication we were following was a published method to synthesize the redox probe, we envisioned a better, more direct route that would allow the entire

synthesis to be complete on solid phase, thus eliminating difficult purifications of side-chain protected peptides and in-solution coupling reactions of functionalities to partially protected peptides.

Molecule **19, figure 2** was produced by solid phase peptide synthesis, as depicted in **Scheme 1**. Using the backbone structure described in Lee et. Al, we modified the peptide to include a 5-Fam/TAMRA FRET pair.[1] From Rink Amide resin, the sequence CTGNVCDap(Dde) was synthesized. The N-terminal cysteine was Fmoc deprotected, revealing a free amine. To the free amine was coupled the 5-Fam fluorophore. Briefly, 1.5 eq of 5-Fam over the peptide on resin, (0.1 mmol scale), 1.5 eq. of HOBT-6Cl, 1.5 eq. of di-isopropyl carbodiimide, and 1.5 eq. DIEA were added to the peptide on resin and allowed to react for two hours. The resin was then rinsed and dried to prepare for the coupling of the second fluorophore.



Scheme 1*:* Synthetic scheme for the production of the redox probe compound **19**, also referred to as "method 1". The entirety of the synthesis is completed on resin.

The Dap residue was then selectively Dde deprotected using hydrazine. A solution of 10% hydrazine in DMF (v/v) was prepared by carefully adding hydrazine to dry DMF. The hydrazine solution is added to the peptide on resin in 3 mL aliquots and allowed to react for three consecutive 5 minute intervals with brief DCM washing steps in between. After the final treatment with the hydrazine, the material was subjected to a Kaiser test to ensure complete deprotection.

Once the Dap sidechain had been determined to be adequately deprotected, the coupling with TAMRA was achieved. TAMRA was purchased as a mixture of its 5-, 6-isomers as an activated ester. The TAMRA activated ester was added to the peptide (0.1mmol) in 1.5 eq, along with 2.6 eq. of DIEA in DMF and allowed to react overnight to ensure complete reaction.

After the TAMRA coupling was complete, the material was cleaved from the resin by Reagent K. The structure of the final product, **19** is shown in Figure 2.

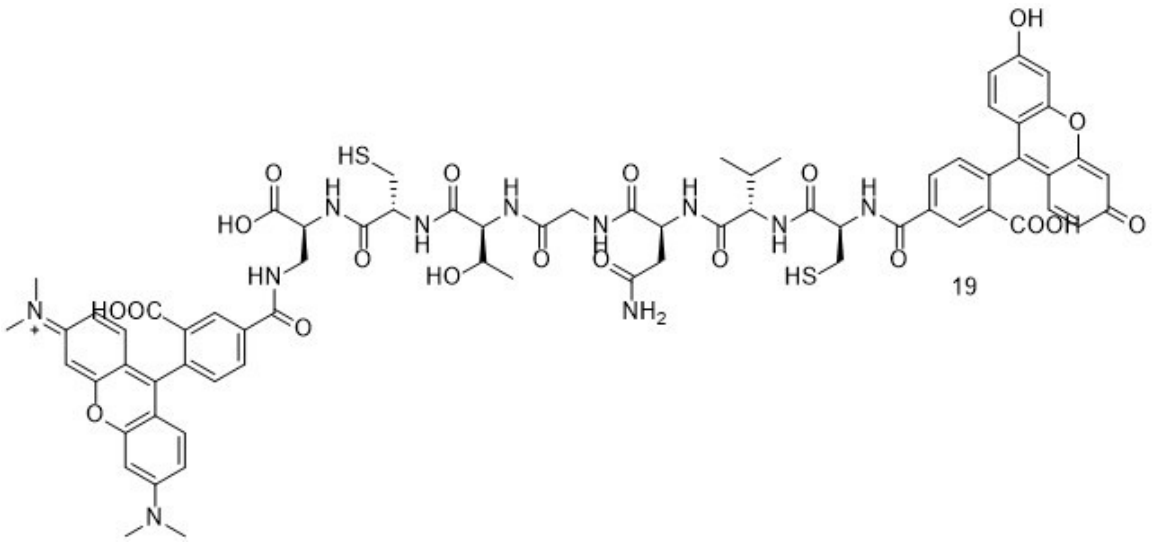


Figure 2: Original planned structure for fluorescence probes; later modified to the structure published by Lee et al.[3]

It was quickly determined that the change in fluorescence upon oxidation and reduction of the probe was not large enough to be useful for our purposes. Therefore, we moved on to modifying the production of the already published structure upon which we based the fluorescence probe.

1.5 Methods: Synthesis of Luminescence based probes (Method 2, Figure 1)

1.5.1 Synthesis of mDTPA chelator, Route 1 (Masuda)[6]

This method was attempted first due to its more recent publication and the fewer synthetic steps required to completion. A limitation of this method is the inability to easily follow the progress of the final coupling of the chelator moiety due to the nature of the

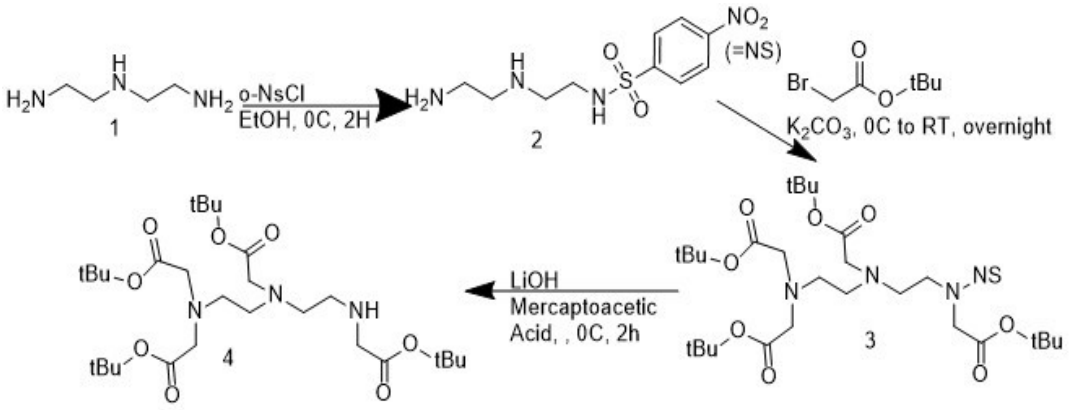
coupling. While the method described in section **1.4.1** lets us monitor progress by colorimetric ninhydrin test, here we do not have the benefit of free amines to quantify.

Diethylenetriamine **1** (5 mmol) was dissolved in dry ethanol and brought below 0˚C. To this solution, o-NsCl (1.5 mmol) was added slowly, keeping the reaction below 0˚C. After 2 hours of stirring at the same temperature, the ethanol was removed by rotary evaporation, revealing crude molecule **2**.

T-butyl bromoacetate (28 mmol) and K2CO3 (35 mmol) were dissolved in dry DMF (10 mL) and added to crude **2** while keeping the reaction below 0˚C. The reaction was allowed to come to room temperature as it stirred overnight. It was then filtered. The filtrate wa concentrated by rotary evaporation and then redissolved in 100mL EtOAc. That solution is washed with brine and dried over MgSO4. The dried material was concentrated by rotary evaporation and purified by flash chromatography (hexanes-EtOAc) to obtain compound **3** (20%) as a yellow oil. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.08–8.11 (1H, m), 7.64–7.69 (2H, m), 7.56–7.60 (1H, m), 4.24 (2H, s), 3.49 (2H, t), 3.42 (4H, s), 3.30 (2H, s), 2.88 (2H, t), 2.78 (2H, t), 2.77 (2H, t,), 1.45 (27H, s), 1.36 (9H, s); MS (ESI-MS) m/z calc. [M+H]+: 746.3, observed, 746.3

Nosyl deprotection of **3** was achieved by dissolving pure **3** (1 eq.) in dry DMF (800 μL). To this solution was added LiOH (10 eq.) and mercaptoacetic acid (5 eq.) while keeping the reaction below 0˚C. After 2 hours, the reaction was concentrated by rotary evaporation and dissolved in methylene chloride. The solution was then washed with brine and saturated bicarbonate and dried over MgSO4. It was purified by flash chromatography with DCM and EtOAc. (yield: 20%) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 3.39

(4H, s), 3.28 (4H, s), 2.72–2.82 (6H, m), 2.63 (2H, t), 1.39 (9H, s), 1.38 (27H, s); MS (ESI-MS) m/z calc. [M+H]+: 560.3, observed, 560.3



Scheme 2: Synthesis of Chelator as described in Masuda et. Al; attempted and modified

1.5.2 Synthesis of mDTPA chelator, Route 2 (Arano)[4]

The second route to the production of the monofunctional DTPA chelator was the Arano et Al method. I initially avoided this method due to the longer synthetic route, but was attracted to the final coupling step of this method. Because the final coupling of the finished chelator is the same amide bond formation reaction employed in the synthesis of a peptide, it opens up the possibility to use the ninhydrin test for free amines to monitor the coupling reaction progress.

In separate containers, ethyl trifluoroacetate (2.37 mL, 20 mmol) was dissolved in 23 mL methylene chloride and diethylenetriamine, (1.57 mL, 20 mmol) was dissolved in 11 mL of methylene chloride. The solution of diethylenetriamine was brought below 0 ˚C with a brine and ice bath. The ethyl trifluoroacetate solution was then added dropwise at a rate of 1-2 mL per minute. Stirring continued below 0 ˚C for 2 hours, at which point the

reaction was allowed to come to room temperature. Stirring continued for an additional 1.5 hours. It was assumed that the reaction to **5 (Scheme 3)** was completed after the cumulative 3.5 hours as suggested in the literature.[6] No MS or NMR data were obtained.

After stirring, the reaction contents were concentrated by rotary evaporation to a pale yellow thin, oily residue. This residue was dissolved in 60 mL acetonitrile and once again brought below 0˚C while stirring. To this flask was added diisoproylethylamine (DIEA, 12.19 mL, 70 mmol) and then t-butyl bromoacetate (10.33 mL, 70 mmol). The reaction was then allowed to come to room temperature while stirring overnight.

The reaction was then concentrated by rotary evaporation to a viscous amber oil. The residue was redissolved in 100mL of ethyl acetate, washed twice with saturated bicarbonate and once with brine before being concentrated once again by rotary evaporation. Purification of this material, compound **6**, **(Scheme 3)** was accomplished by flash chromatography (r.f. of product 0.43, 1:4 ethyl acetate in hexanes; 28% yield). ^{1}H-NMR ($CDCl_3$): δ 8.59 (1H, s), 3.44 (4H, s), 3.33-3.38 (2H, m), 3.29 (2H, s), 2.83-2.88, 2.75-2.79, (6H, m), 1.45 (27H, s); MS (ESI-MS) m/z calc. $[M+H]^+$: 542.1, obs. 542.1

Purified **6** (3 g, 5.54 mmol) was dissolved in dry dimethylformamide and brought to below 0˚C in a brine and ice bath. Separately, ~500 mg of 60% sodium hydride dispersion in paraffin was dissolved in 10 mL of dry dimethylformamide. The sodium hydride was added carefully to the stirring **6** in DMF, keeping the temperature below 0˚C. Stirring continued for 30 minutes below zero and then 30 minutes at room temperature while hydrogen gas vigorously evolved. After bubbling ceased, the reaction was once again brought to below 0˚C and t-butyl bromoacetate (1.22 mL, 8.31 mmol) was added. After

addition of the alkyl bromide the reaction was allowed to come to room temperature while stirring overnight.

The next day, the reaction was poured into 200 mL of ethyl acetate and washed twice with saturated sodium bicarbonate and once with brine. It was then concentrated in vacuo to a thick dark yellow oil, crude **7**. Purification of this material was accomplished via flash chromatography (r.f. of product 0.50 in 20% ethyl acetate in hexanes, 70%). ^{1}H-NMR ($CDCl_3$): δ 4.29 (1H, d), 4.16 (1H, s), 3.55 (2H, m), 3.42 (4H, s), 3.31 (2H, s), 2.76-2.79, 2.85-2.92 (6H, m), 1.45-1.47 (36H, s). MS (ESI-MS) m/z calc. $[M+H]^+$: 656, obs. 656

The purified **7** (2 g, 3 mmol) was dissolved in 8 ml of t-butanol. Once dissolved, this solution was brought to below 0˚C with an ice/brine bath. This solution will freeze solid. At this point, 100 molar equivalents of anhydrous hydrazine were added. The solution was allowed to stir, maintaining the 0˚C temperature, for 3.5 hours.

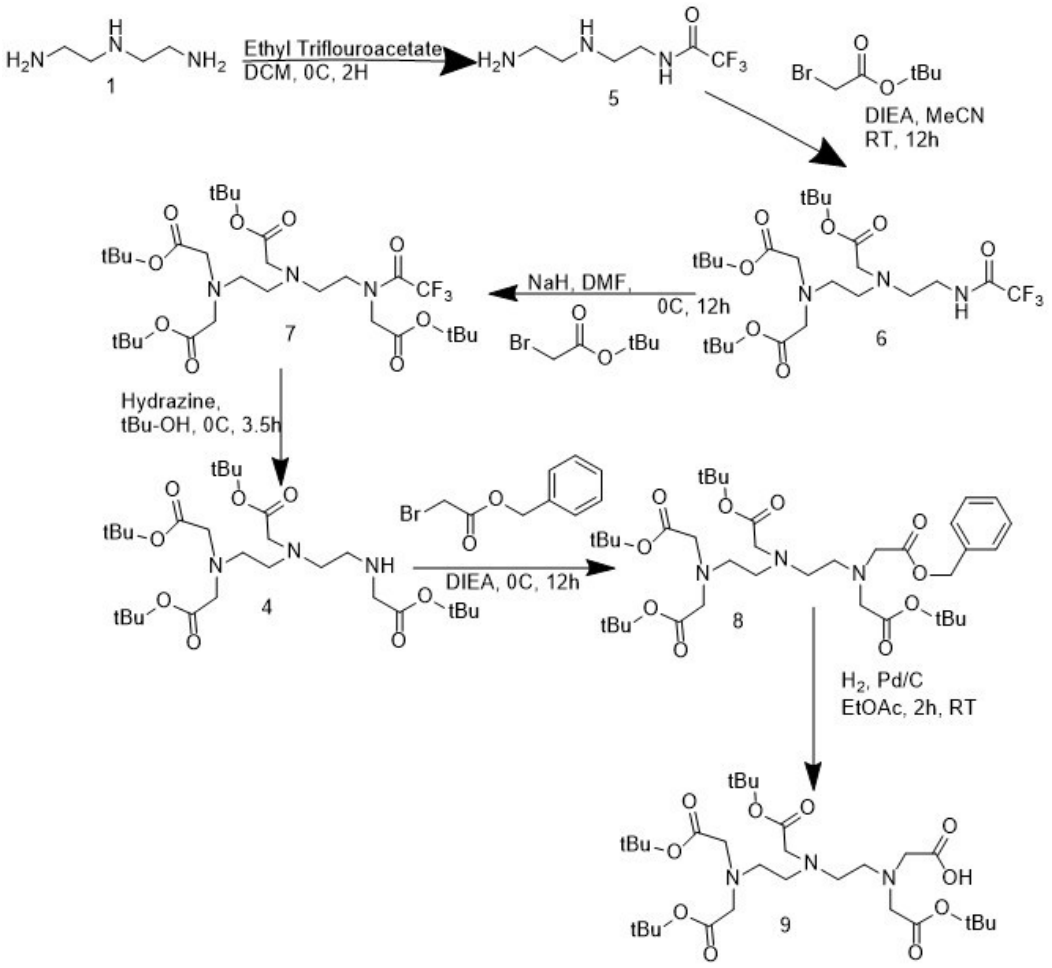
After 3.5 hours, the hydrazine reaction mixture was added to 150-200 mL of methylene chloride and washed twice with saturated bicarbonate and once with brine. It was then dried over magnesium sulfate and concentrated, giving crude **4**. No purification or characterization was completed at this stage, but TLC data using 1:1 hexanes:ethyl acetate indicated near quantitative conversion.

Crude **4** was then dissolved in 3mL dry DMF and brought below 0˚C once again. To this solution is added DIEA (4 eq.), followed by benzyl bromoacetate (4 eq.). After the addition of the benzyl bromoacetate the reaction was allowed to come to room temperature and stirring continued for at least 12 hours.

Ethyl acetate (10x the volume of the DMF) was added to the reaction, and washed twice with saturated bicarbonate and once with brine. The material was then concentrated by rotary evaporation before purification via flash chromatography, giving compound **8** (Scheme 3, (r.f. 0.6, 1:5 hexanes: ethyl acetate, 5%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.33-7.36 (m, 5 H); 5.14 (s, 2 H), 3.64 (s, 2 H), 3.46 (s, 2 H), 3.43 (s, 4 H), 3.33 (s, 2 H), 2.74-2.86 (m, 8 H), 1.44 (s, 36 H), MS $[M+H]^+$ obs. 708; calc. 708.

Compound **8** (150 mg, 0.2 mmol) was dissolved in ethyl acetate (5 mL) at room temperature. Benzyl deprotection to reveal the reactive carboxylic acid was achieved by catalytic hydrogenation using Pd/C and H_2 gas. Anywhere from 100-250 mg of Pd/C was added to the dissolved **8** while stirring, and a hydrogen filled balloon was secured on top of the reaction vessel. The reaction proceeded at room temperature for 6 hours.

The reaction was then filtered through celite to remove the solid Pd/C and concentrated by rotary evaporation. The benzyl deprotected material should be used within a day or so of deprotection and it should never be stored in solution. No NMR data were obtained at this stage, but MS data were generated to ensure complete benzyl deprotection was achieved. MS (ESI) m/z $[M+H]^+$ obs. 618; calc. 618.



Scheme 3*:* Synthesis of mono-functional DTPA as described in Arano et. al, modified as described.

After completing the synthesis of the chelator using both methods, it was determined that the best course of action was to use a method that combines the best features of both. In the Arano et. al methodology, all reactions up until the final alkylation proceeded without complication. The final alkylation, (the reaction from **4** to **8**) however, proved problematic. It was the poor yield at this stage that motivated us to decide to stop

the synthesis at compound **4**, the common intermediate of both the Arano and Masuda methods, and attempt the Masuda-style coupling to the peptide on resin.

This would prove successful.

1.5.3 Synthesis of Carbostyril 124[8]

The synthesis of the Carbostyril 124 (**10**) sensitizer moiety was achieved as described in the referenced publication, Woods et. al.[8] In a single flask, equimolar amounts of m-phenylene diamine (21.2 mL, 0.166 mmol) and ethyl acetoacetate (18.0 g, 0.166 mmol) were combined and agitated until all solid was dissolved. The solution was heated to reflux with an oil bath at 150˚C for 18 or more hours. The reaction formed a chunky, dark brown solid. Water was added to this crude mixture and brought to boiling to break up the chunks. After an additional several minutes of boiling, the mixture was filtered and the solid was retained and air dried.

Purification of **10** was accomplished by recrystallization from absolute ethanol. (yield, 40%). ^{1}H-NMR (DMSO, 300 MHz) δ 11.17 (1 H, s), 7.37 (1H, d), 6.49 (1H, dd), 6.43 (1H, s), 5.99 (1H, s), 5.71 (2H, s), 2.31 (3H, s).

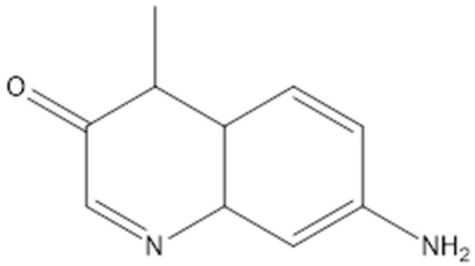


Figure 3: Carbostyril 124, molecule **10**

1.5.4 Synthesis of Cystiene-CS124 (11)

Boc-Cys(Acm)-OH (1 eq.) was dissolved in DMF (5 mL). Separately, Carbostyril 124 (1.2 eq.) was dissolved in DMF (5 mL). To the cysteine solution was added 1 eq. each of isobutyl chloroformate and n-methylmorpholine. The cysteine solution was allowed to activate for 10 minutes on ice. After the allotted time, the carbostyril solution was added to the activated cysteine. The reaction was allowed to proceed on ice for 30 minutes, and was then brought to room temperature while stirring overnight. ^{1}H NMR (DMSO, 400 MHz) δ 11.59 (1H, s), 10.25 (1H, s), 7.77 (1H, d), 7.64 (1H, d), 7.34 (1H, dd), 7.22 (1H, d), 6.25 (1H, s), 4.27-4.31 (3H, m), 2.89-2.93 (1H, m), 2.73-2.79 (1H, m) 2.36 (3H, s), 1.86 (3H, s), 1.38 (9H, s).

1.5.5 Attempts at Cys-CS124 (11) to chlorotrityl resin.[7]

In an effort to simplify the synthesis of this probe molecule, I envisioned a way in which to complete the condensation of all three pieces (peptide backbone, antennae and chelator) while the probe was on resin. This is described in Method 2, **Figure 1**.

I looked to the publication of out the Distefano group from 2012, Diaz et al, for inspiration. The publication references a method to use a thioether, side-chain linkage for the C-terminal cysteine. This leaves the C-terminal carboxylic acid free to be functionalized. I attempted the methodology described in the paper on multiple occasions, including an adaptation for microwave synthesis. None of the attempts achieved sufficient resin loading to proceed with a peptide synthesis from the resin. The data from the 2012 Diaz et. Al publication supports what I found. Namely, the addition of a more sterically hindered C-terminal moiety decreases loading reaction efficacy. The carbostyril moiety is

the largest known attempted C-terminal functionality for a C-terminal cysteine sidechain loading methodology, and though it is disappointing that the strategy failed, it is not surprising. I moved on to the methods reported here.

1.5.6 Synthesis of probe backbone (12)

The CTC resin was swollen in DCM for 15-30 minutes. Fmoc-Thr(tBu)-OH (1 eq.) and DIEA (5 eq.) was added to the resin. The reaction proceeded for 5 minutes at room temperature, at which time an additional 1.5 equivalents of DIEA was added. The reaction was allowed to proceed overnight at room temperature on a rotisserie. After reaction was complete, 20 μL of methanol were added to the reaction mixture to end-cap any remaining active sites on the resin. The endcapping proceeded on rotisserie for an additional 5 minutes. The resin was rinsed 3x with DCM, then DMF, then DCM and stored in a desiccator. The resin loading was analyzed by Fmoc cleavage and analysis.

When resin loading was determined to be 0.3 meq/g of resin or greater, the synthesis of the peptide backbone proceeded by manual Fmoc solid phase peptide synthesis.

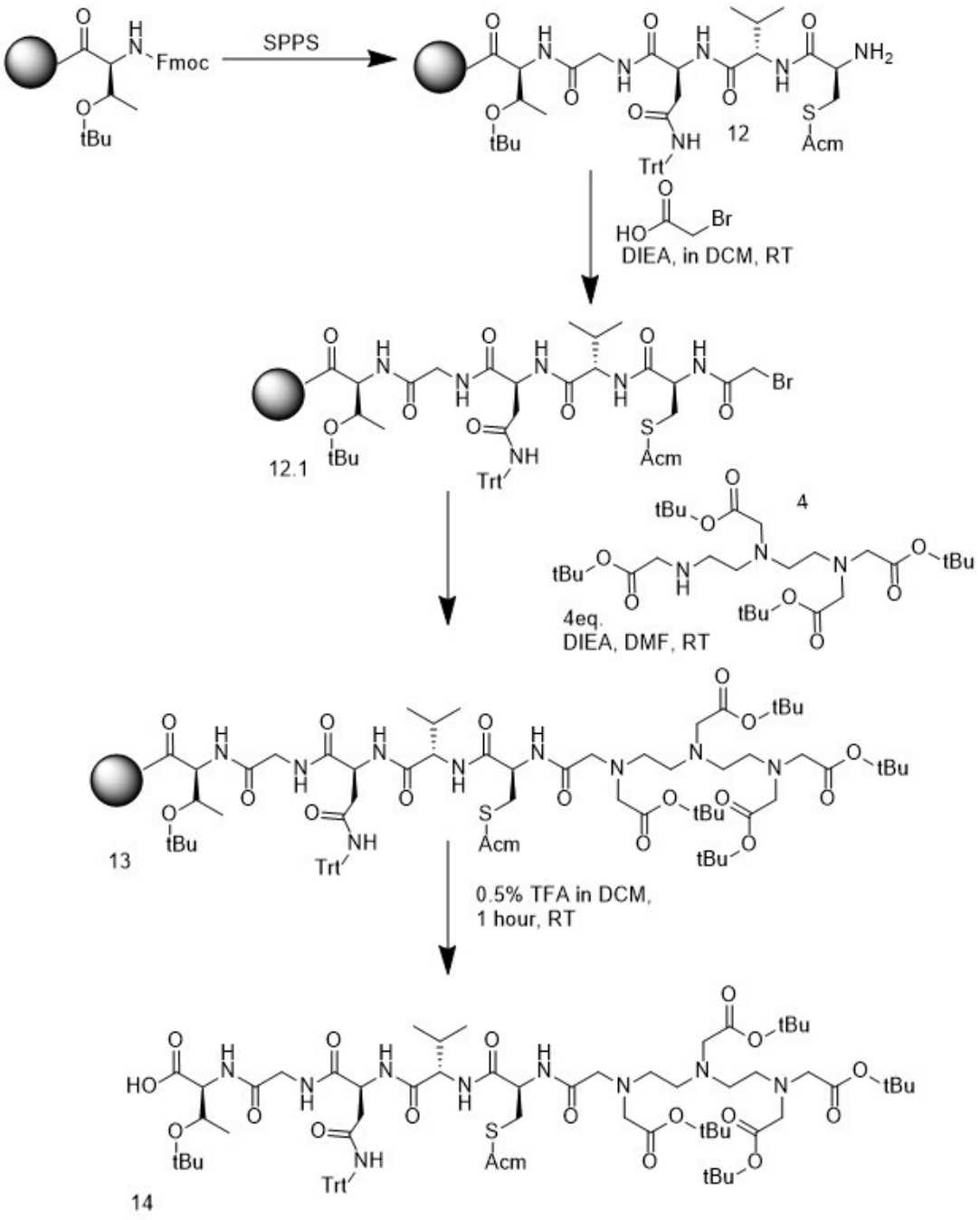
1.5.7 Attachment of mDTPA (4) to probe on-resin (12) to give Compound 13.

Bromoacetic acid (1 eq. in relation to the peptide, 80 mg) and DIEA (75 μL) were added to the resin swollen in DCM (50 mg, approximately 0.65 meq/g substitution) and allowed to react at room temperature on the rotisserie for 45 minutes to an hour. At this point, the resin was washed with DCM, MeOH, and DMF. The material was subjected to a ninhydrin test to confirm the coupling had gone to completion. If the ninhydrin test was

negative, the resin was dried and reserved for the next step. The coupling sometimes needed to be completed more than once.

Compound **4, Scheme 4,** (0.13 mmol, 4 eq.) was dissolved to a concentration of 1 mg/μL in DMF and added to the bromoacetylated peptide (12.1, Scheme 4) along with DIEA (4 eq). After 12 or more hours, the peptide on resin was washed thoroughly with DCM, MeOH and DMF to remove as much of the uncoupled chelator as possible. Excess chelator will complicate the mass analysis of this material, as it ionizes much more readily than the desired product. Cleavage of **13** to **14** was accomplished by treatment with 0.5% TFA in DCM for one hour at room temperature. The residue obtained from this was evaporated to an oil under a stream of nitrogen, dissolved in a 1:1 solution of acetonitrile and water with 1% acetic acid, flash frozen, and then lyophilized to a white powder (**14**). The preceding reactions are depicted in **Scheme 4**.

Purification of **14** was completed by C-18 semi-preparative HPLC. (r.t. 36 min, 0-100%B, 1% min gradient). MS (ESI) m/z $[M+H]^+$ obs.1461.9; calc. 1461.7



Scheme 4: Synthetic scheme showing the production of compound **14**, starting from compound **12.**

1.5.8 Attachment of Cys-CS124 (11) to (14), to give Compound 15.

Compound **14** (10mg, 6 μmol) was dissolved in DMF and cooled to below 0˚C. To this solution, isobutyl chloroformate (1.5 μL) and n-methylmorpholine (1 μL) were added. The resulting reaction was allowed to proceed for 10 minutes, at which time (**11**, 2 eq), dissolved in a minimum volume of DMF, was added to the solution. The reaction was maintained at a temperature of 0˚C for 1.5 hours. At this point, the reaction was allowed to come to room temperature and was allowed to proceed on the rotisserie at room temperature for anywhere from 2 to 24 hours. The next step was undertaken without further purification. (r.t. 15 min, 0-100%B, 2% min gradient). MS (ESI) m/z $[M+H]^+$ obs. 1791.4; calc. 1791.6

1.5.9 Intitial deprotection of (15) to afford (16).

Compound **15** was treated with a solution of 98:2 TFA:thioanisole for one hour. After 1 hour, the TFA was evaporated under a stream of nitrogen gas. After the TFA was removed, the resulting residue was dissolved in 1:1 water:acetonitrile, 1% acetic acid, flash frozen in liquid nitrogen, and then lyophilized to a white solid. The material was unable to be purified by C18 semi-preparative HPLC and was used in the next reaction in a partially crude form. (r.t. 56 min, 0-100%B, 1% min gradient). MS (ESI) m/z $[M+H]^+$ obs. none; calc. 1269.5

1.5.10 Acm deprotection of (16) to afford (17).

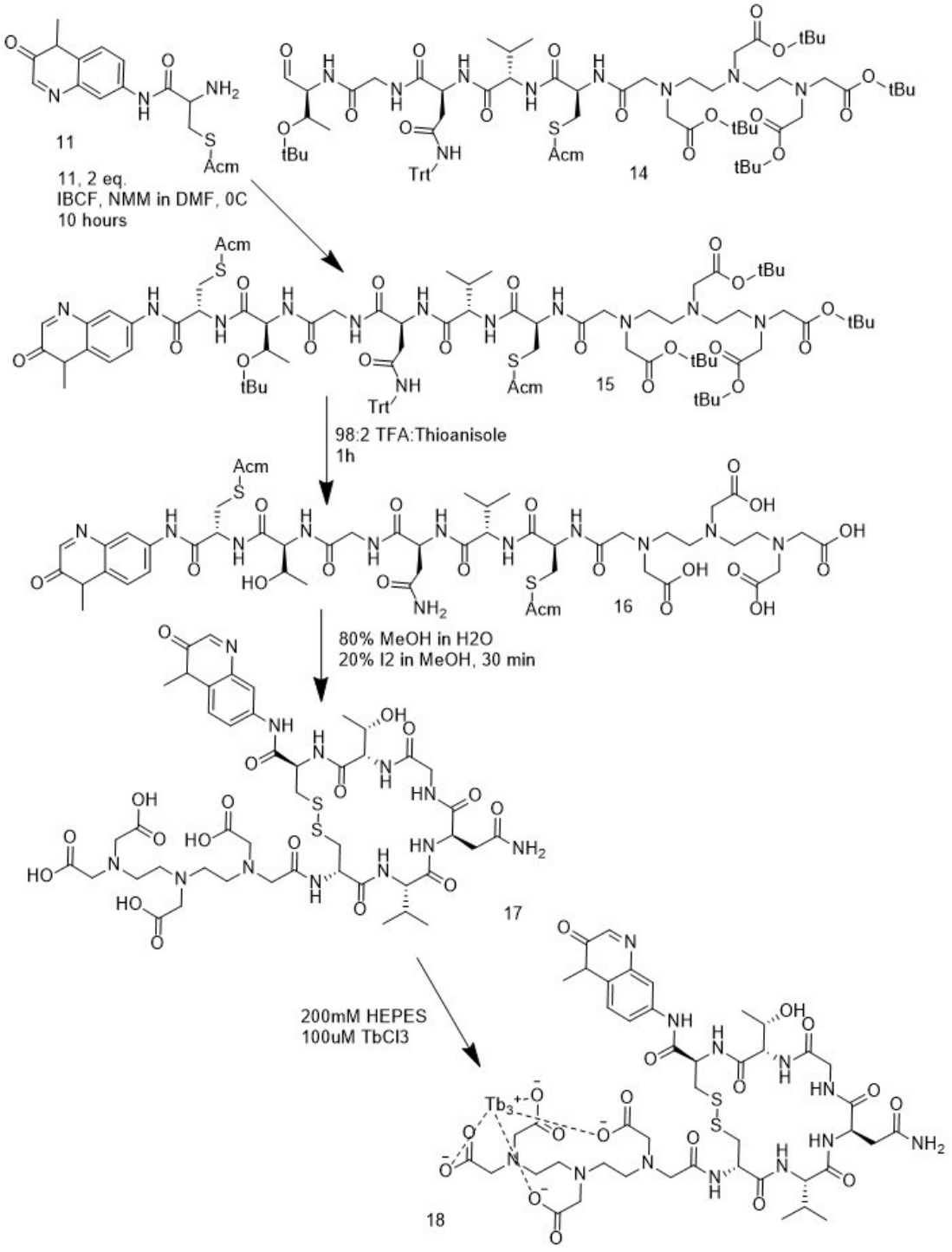
Compound **16** (10 mg, 7.8 μmol) was dissolved in 50 mL of an 80% methanol in water solution. To this solution, 185 μl of a 20% I_2/methanol(w/v) solution was added to the

compound **15** solution and stirred for 30 minutes. The solution at this point is a dark, cloudy brown color. After 30 minutes, the reaction is quenched with 500 µl of a 1M ascorbic acid solution, at which point the brown color disappears, resulting in a clear, slightly cloudy solution. This solution was concentrated by rotary evaporation until 5 mL remained.

Purification of **17** was completed by C18 semi preparative HPLC. (r.t. 23 min, 0-100%B, 1% min gradient, C4 analytical). MS (ESI) m/z $[M+H]^+$ obs. 1125.4; calc. 1125.4

1.5.11 Metallation of DTPA-Cys-124 to afford (18).

Compound **17** was dissolved in HEPES buffer (200mM HEPES, 150mM NaCl, pH 7.0) and to this solution was added 2µL of a solution of TbCl3 (100µM). The reaction was stirred for 30 minutes. Compound **18** was found in the crude material by MALDI-MS, but the final crude mass of this reaction was so low that no purification was attempted. The preceding reactions are depicted in **Scheme 5**. (r.t. 14.2 min, 0-100%B, 2% min gradient) MS (ESI) m/z $[M+2Na]^+$ obs. 1325.2; calc. 1325.2



Scheme 5: Synthetic scheme showing the production of the redox probe, from molecule 14 through to the final product, molecule 18.

1.6 Results, Conclusions and Future Work.

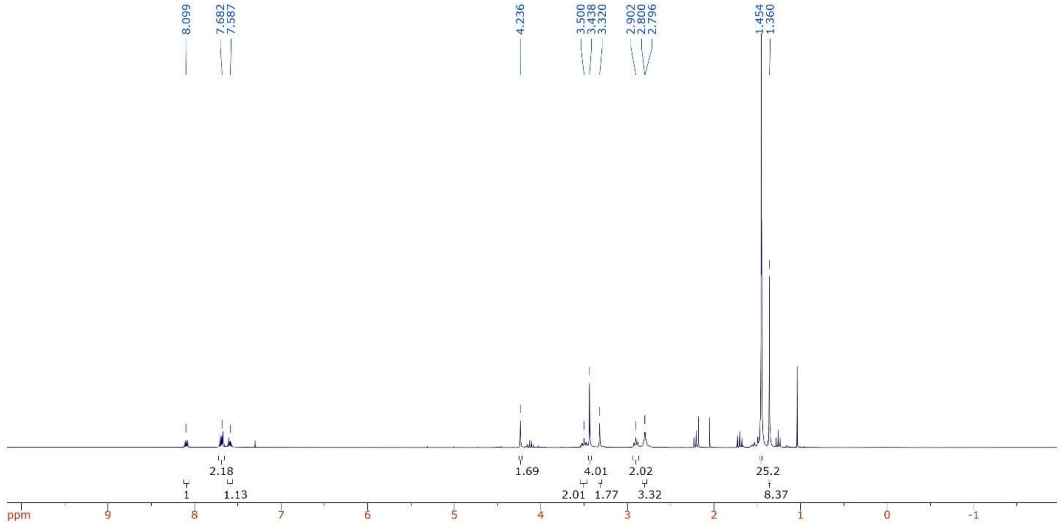
After several years of attempts at the complete synthesis of this probe from a variety of angles, some of the worst bugs of the synthesis, namely the production of the chelator and the coupling of that chelator to the peptide on resin, have been worked out. By using a hybrid of the Arano and Masuda synthesis methods, I developed what I believe to be a better route to the chelator, and that allowed us to scale up the reaction. Specifically, the best route to a successfully coupled chelator was found to be the use of the Arano methodology to the secondary amine (**4**), followed by application of the Masuda method's protocol for coupling the protected, monofunctional chelator to the bromoacetylated peptide on resin **(12.1)**. While it was initially thought that the Arano method for coupling to the peptide on resin (amide bond formation between **9** and unbromoacetylated peptide on resin **12** was superior due to the ability for the coupling reaction to be followed via ninhydrin test, it was determined that the synthetic difficulties of obtaining **8,** and **9** in sufficient yield were too limiting.

I unfortunately had to abandon the idea of using a completely on-resin methodology to produce the published structure **(Figure 1, Method 2)** due to exceptionally poor coupling efficiencies of the carbostyril functionalized C-terminal cysteine moiety to the resin. However, I successfully produced the redox probe using the in-solution couplings described in the Lee et. Al paper. At present, the methods are sufficiently robust to bring the production of the material to larger scales, perhaps even in an undergraduate laboratory.

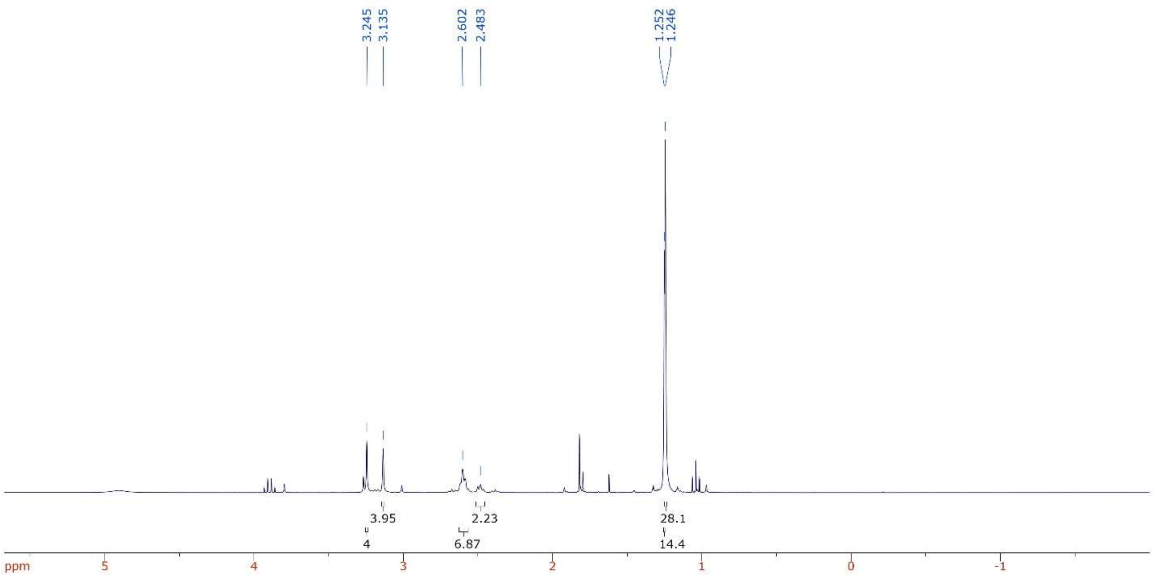
Plans are in place to implement the methods in future research endeavors with undergraduate students.

Future applications of the synthesized probe include quantitative analysis of redox environment changes in biological fluids (blood, cerebral spinal fluid) associated with increased ROS production in disease states. The method proposed here presents an advantage to currently available methods in that it does not require endogenous expression, which makes it applicable to primary cell and tissue analysis. It also presents the possibility for point-of-care diagnostics to track onset, progression and status of redox-related diseases.

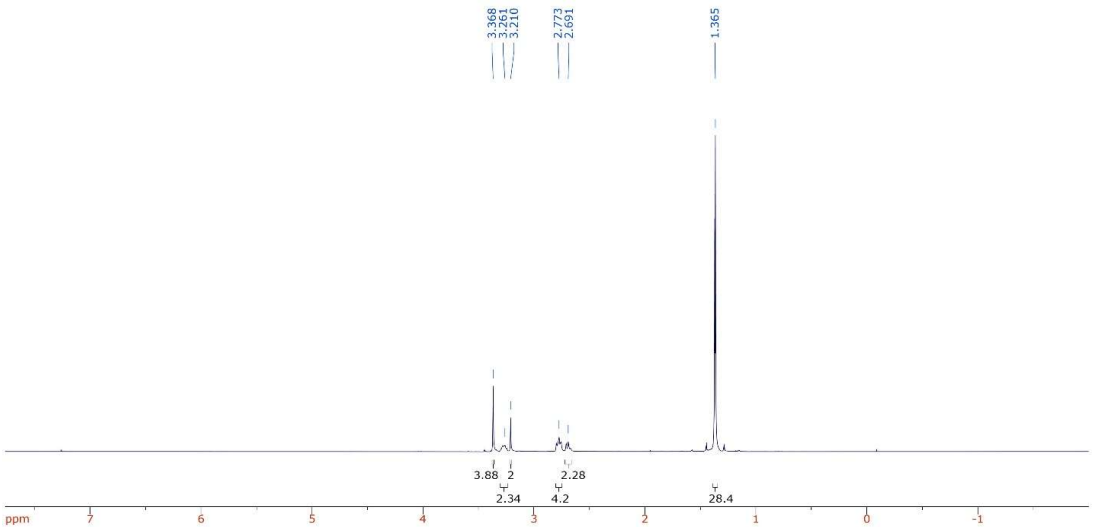
1.7 Supporting Information



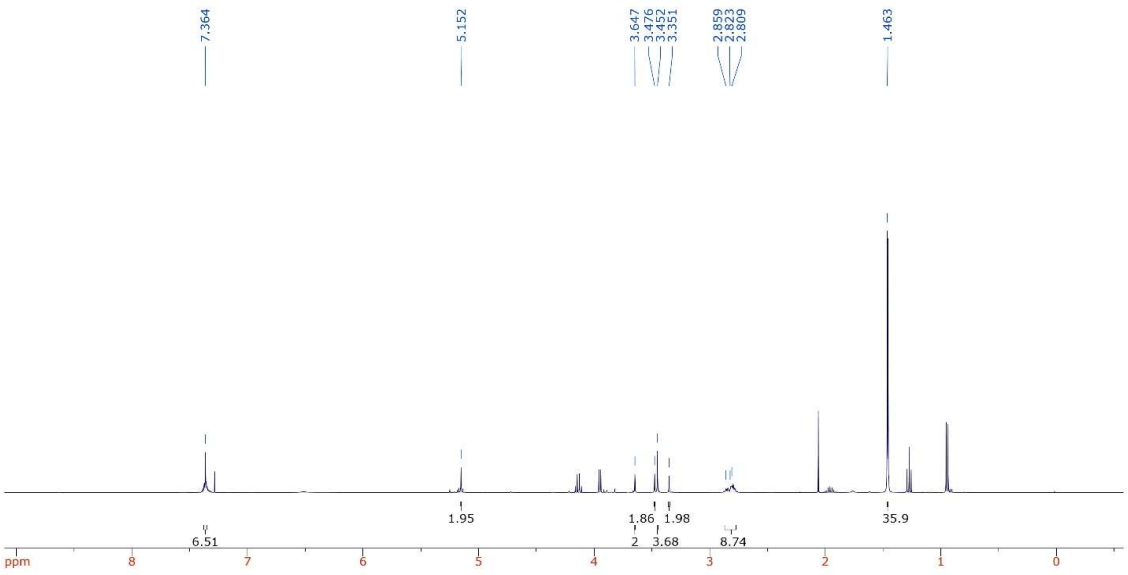
S 1 Compound **3** NMR, 300 MHz, $CDCl_3$



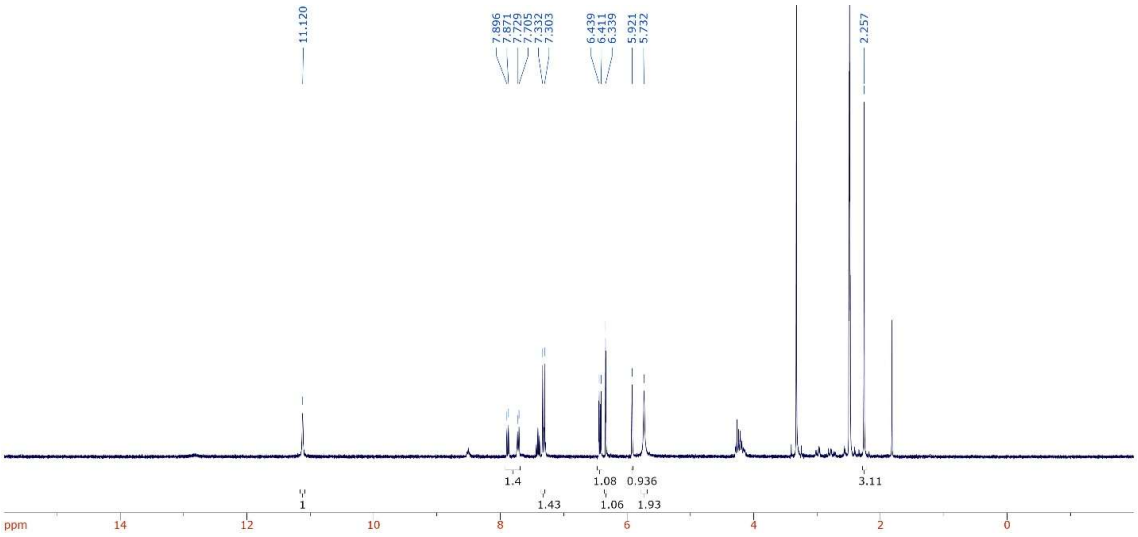
S 2 Compound **4** NMR, 300 MHz, $CDCl_3$



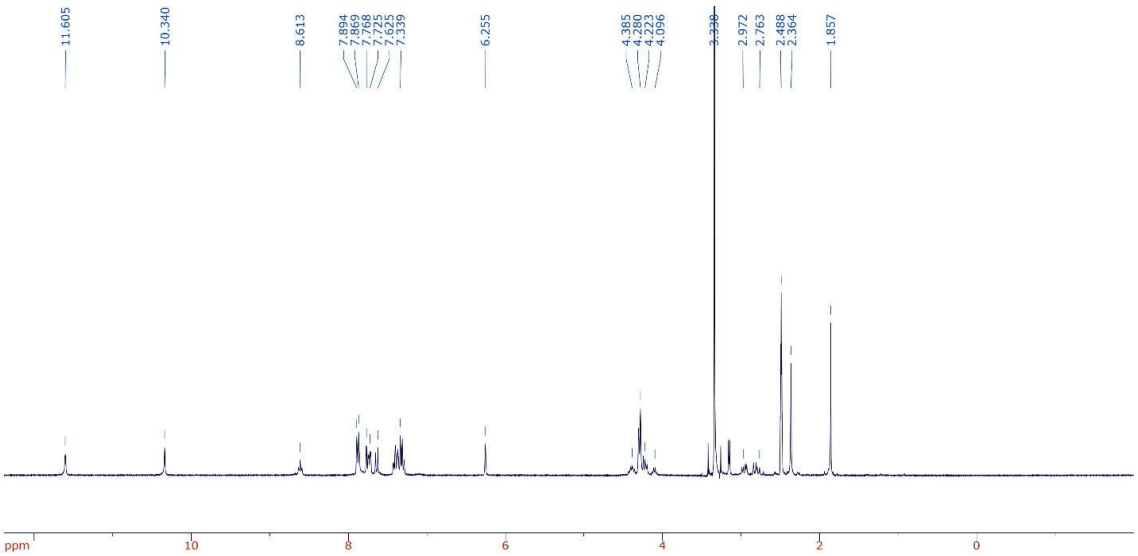
S 3 Compound **7** NMR, 300 MHz, $CDCl_3$



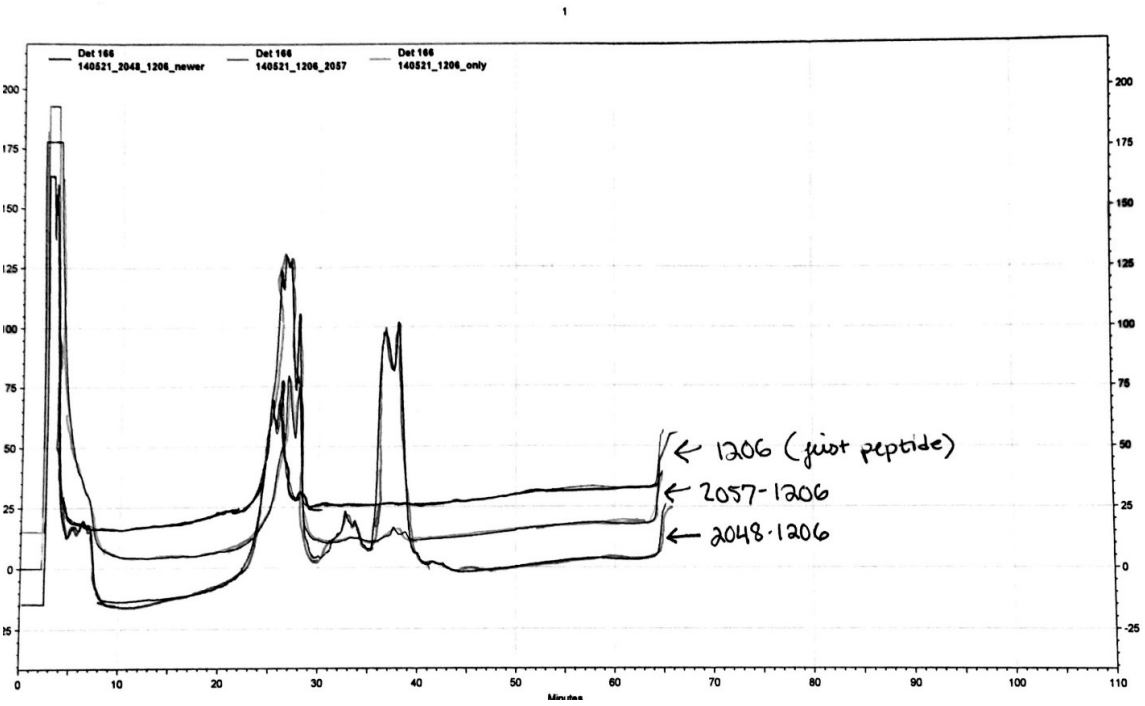
S 4 Compound **8** NMR, 300 MHz, $CDCl_3$



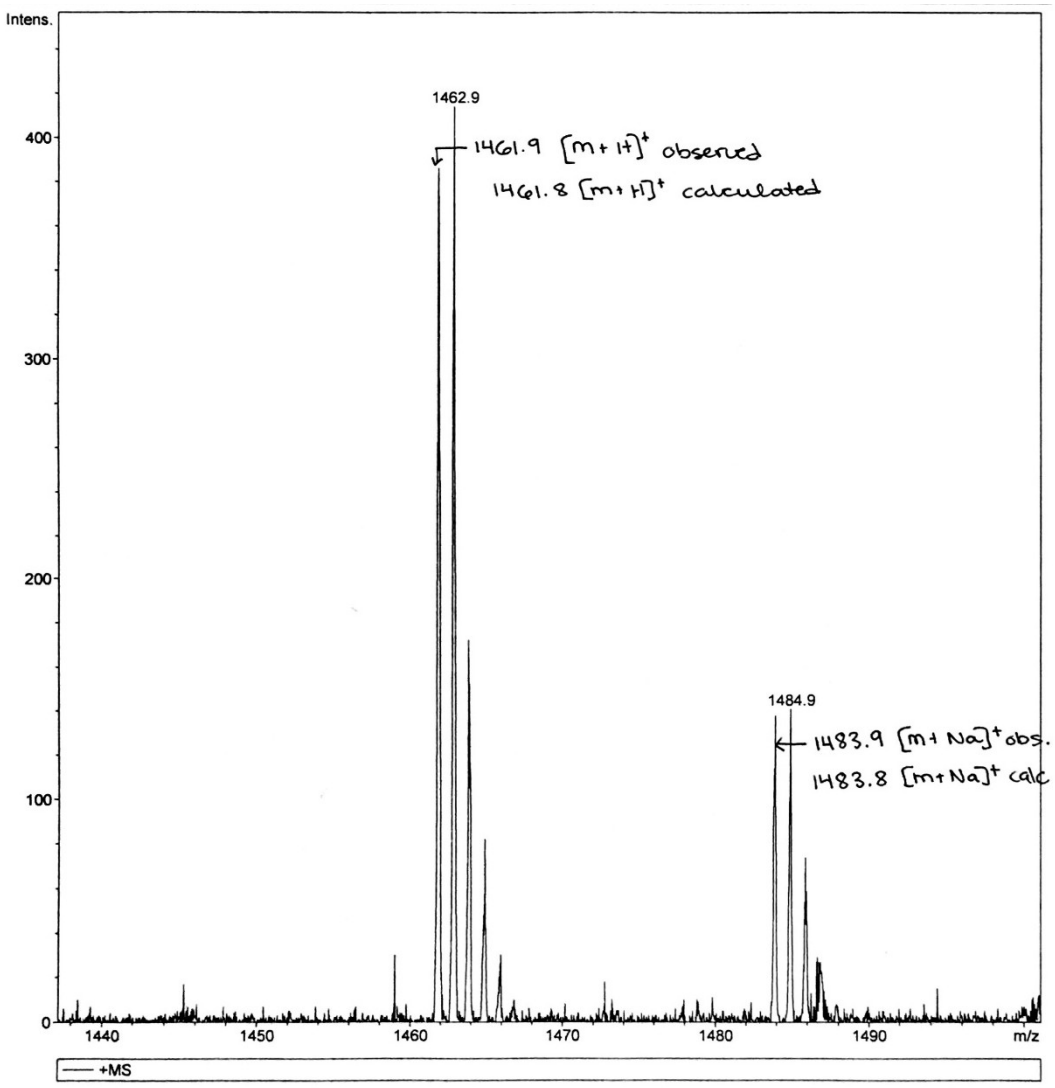
S 5 Compound **10** NMR, 300 MHz, $CDCl_3$



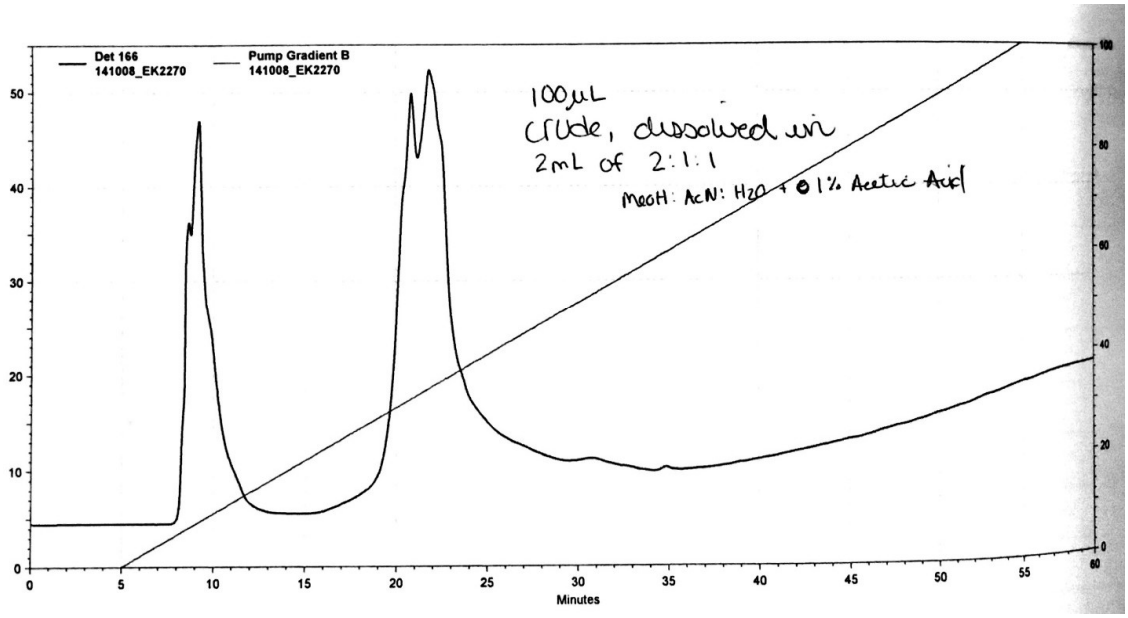
S 6: Compound **11** NMR, 300 MHz, $CDCl_3$



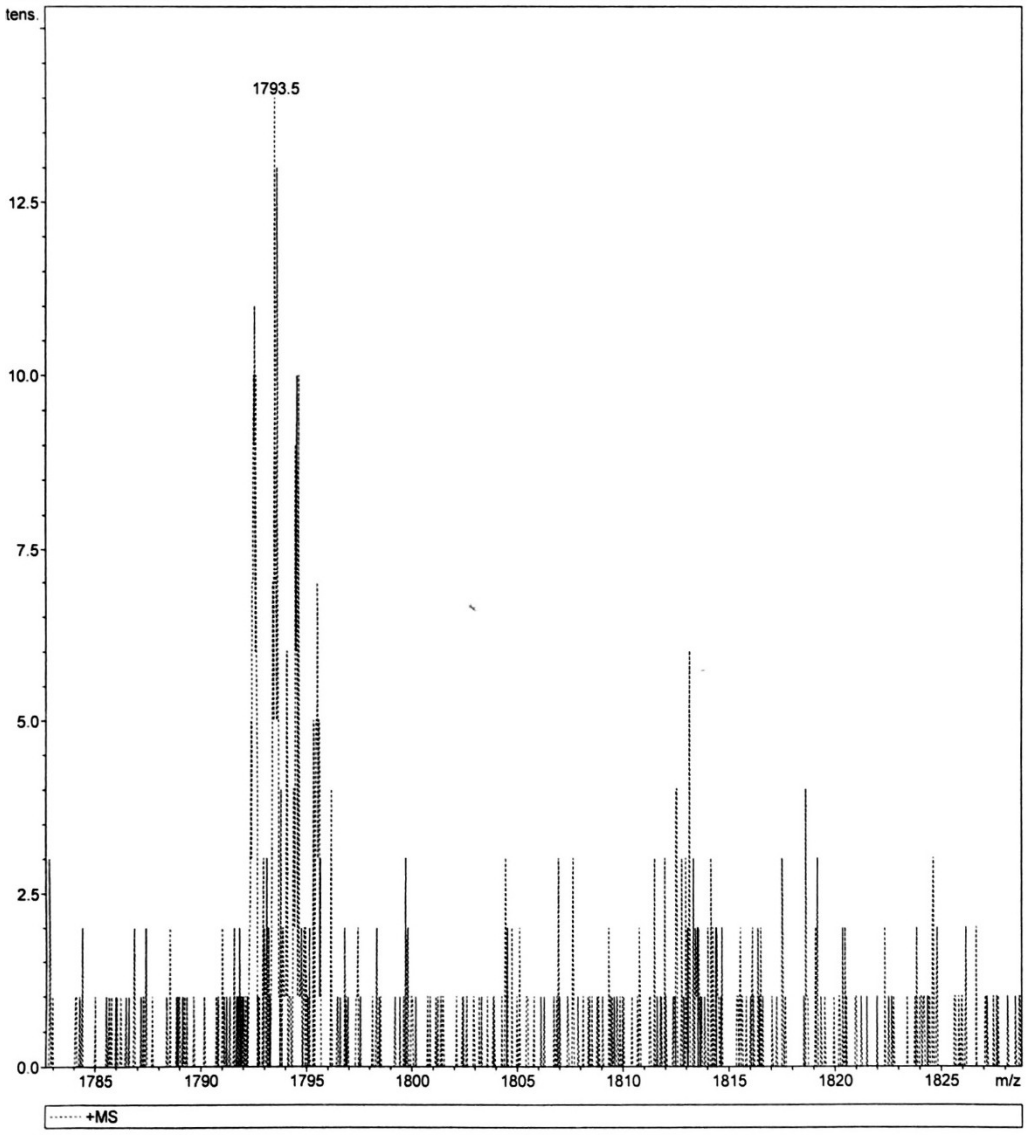
S 7 HPLC trace showing the progression of the coupling of **4** to **13** on resin, resulting in compound **14**. Small amounts of material were cleaved by 0.5% TFA in DCM at 30 minutes of reaction and then 4 hours of reaction to examine reaction progress.



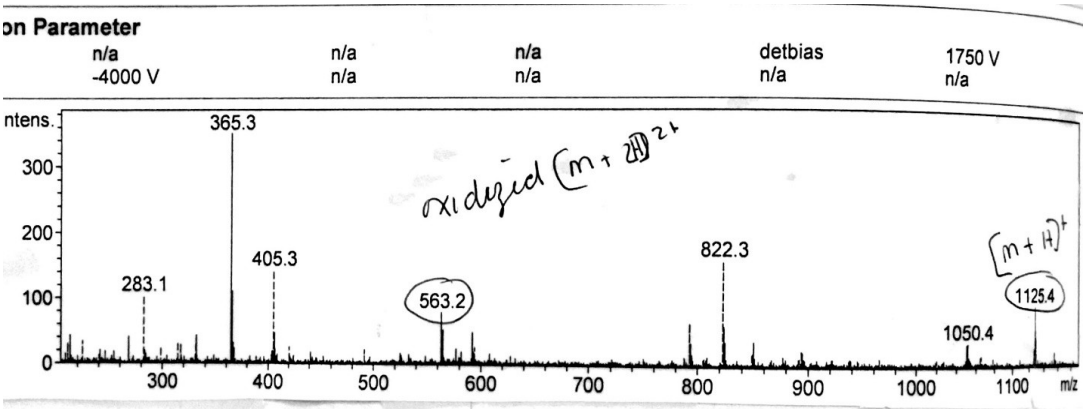
S 8 ESI-MS, + mode, Compound **14**. Compound **14** was dissolved in a 1:1 acetonitrile:water, 1% acetic acid solution and 100 µl of this solution was injected.



S 9 Compound **15,** Crude. C18 HPLC, analytical. Injections of 50 µL of each sample were completed. Data shown were collected at 220nm, with a gradient of 5-60% B at a 1%/ min gradient.



S 10 ESI-MS, + mode, Compound 15. Compound 15 was dissolved in a 1:1 acetonitrile:water, 1% acetic acid solution and 100 µl of this solution was injected.



S 11 Compound **17**, oxidized. ESI-MS, positive mode. Compound **17** was dissolved in a 1:1 acetonitrile:aater, 1% acetic acid solution and 100 µl of this solution was injected

Chapter 2: Synthesis and Analysis of 33-Residue Peptide Substrates Suitable for the Analysis of the Upstream Cleavage Site Catalyzed by Ste24p.

2.1 Abstract

Maintenance of the natural (L)- stereochemistry of amino acids during peptide synthesis and their incorporation into peptides and proteins is critical to the successful synthesis of biologically relevant and active compounds. Most amino acids are resistant to racemization under the conditions used for Fmoc solid phase peptide synthesis. Furthermore, the commonly used activators including HCTU and HOBT actively assist in ensuring the preservation of the appropriate epimer upon the completion of the new amide bond.[9]

However, epimerization can occur in some instances. Here, I examine the specific case of a peptide with a C-terminal cysteine.[10] During Fmoc solid phase synthesis, sequential treatments with base during the Fmoc-deprotection step presents repeated chances for the abstraction of the C-terminal cysteine's α-proton. Subsequent reprotonation can be essentially random and can occur to yield either orientation (L- or D-), which results in a epimeric mixture of final product. A mixture of peptides with a single epimerized amino acid is frequently impossible to separate or even identify by conventional HPLC methods. In particular, when the peptides being synthesized are to be used in biological assays, complete characterization of the material is critical for a thorough understanding of the activity since it is possible that an epimerized substrate may not behave the same way as its correct counterpart.

In addition to the racemization studies, I synthesized some additional analogues of an internally quenched cleavage probe to study the response of yeast Ste24p's N-terminal cleavage activity to changing C-terminal functionalities. Ste24p is a protease involved in the post-translational processing of yeast mating hormone, **a**-factor.[11] It completes endoproteolysis of the immature pheromone at two sites, first near the C-terminus of the protein and the second 16 amino acids upstream. The natural substrate of this enzyme features a methyl ester at the C-terminus of the peptide. Synthesis of C-terminally functionalized probes can be challenging, so I sought to determine whether the C-terminal functionality mattered for the enzyme's activity. Additionally, I endeavored to learn more about the kinetics and the apparent promiscuity of Ste24p's N-terminal cleavage activity.

I synthesized four structures, one containing the native C-terminal methyl ester, one with a C-terminal acid, one with a C-terminal amide and, finally, a D-cysteine methyl ester. While at the time of this writing, the D-cysteine methyl ester cleavage data was pending, I can make the preliminary conclusion that the C-terminal functionality does not appear to affect the cleavage kinetics of yeast Ste24p.

2.1 Background

Various synthetic modifications have been developed to address this issue with C-terminal cysteine peptides, but their effectiveness is not absolute.[11-15] In 2015, Diaz-Rodriguez et. al. demonstrated that when using a cysteine side-chain anchoring methodology to synthesize c-terminal cysteine peptides, peptides containing up to 10 amino acids are free from detectable racemization.[7] In the 2015 publication, NMR

experiments were employed to probe and quantify the extent of racemization by first synthesizing the unfarnesylated L- and D- versions of a truncated yeast hormone peptide known as **a**-factor. In this thesis, the same side-chain anchoring methodology, depicted in **Scheme 7**, shown to be effective at minimizing racemization of 10-mer peptides is explored for its utility in the synthesis of epimer-free peptides 33 residues in length.

Protein prenylation involves the attachment of C_{15} and C_{20} isoprenoids to proteins. This modification occurs on specific cysteine residues positioned within a tetrapeptide CAAX-box sequence localized near the C-termini of a number of proteins and is catalyzed by protein prenyltransferases that link an isoprenoid moiety to the thiol group of cysteine to form a thioether bond. In the CAAX-box, "C" represents the cysteine residue (site of modification), A indicates variable residues and X is a residue that determines whether a farnesyl (C_{15}) or geranylgeranyl (C_{20}) group is added. Prenylation is typically followed by proteolytic cleavage of the AAX tripeptide and subsequent methylation of the resulting carboxylate to form a methyl ester. In yeast, the proteolytic reactions are catalyzed by either Rce1p or Ste24p while methylation is performed by Ste14p; mammalian cells contain homologs with similar function.[12-14]

In addition to cleavage of a C-terminal AAX tripeptide at the C-terminus of a prenylated protein, yeast Ste24p also catalyzes cleavage at very different sites as well. In the maturation of a-factor, a dodecapeptide pheromone from yeast, Ste24p is responsible for the proteolysis of a precursor peptide that has been prenylated, proteolyzed and methylated, as described above. In this case, the cleavage occurs at a position 14 residues upstream from the prenylated cysteine; thus Ste24p is responsible for the hydrolysis of two

different amide bond linkages that have no significant structural similarity. To better understand that process, I elected to synthesize peptide substrates that could be used to study some of the structural requirements of the upstream cleavage site. In particular, I prepared 33-residue peptides that incorporate a donor quencher pair (Abz and 2,4-DNP in this case) that could be used in a continuous spectrofluorimetric assay to monitor Ste24p cleavage activity. Here, I describe the preparation and use of peptides that contain different forms of the C-terminal farnesylcysteine residue including a C-terminal methyl ester, acid and amide. For the methyl ester substrate, peptides where the stereochemistry of the C-terminal farnesylcysteine varied (L- or D-Cys) were also examined.

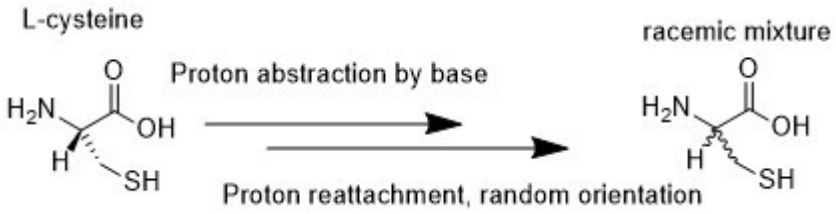


Figure 3: Example of a cysteine's α-proton getting abstracted by base. The proton reattachment can occur in random orientation, resulting in a racemic mixture of product.[15]

I then connected this epimerization phenomenon to work that was being done to better characterize the process of protein prenylation. Protein prenylation is just one of a variety of post translational modifications (PTMs) in eukaryotic biological systems. As their title suggests, PTMs are modifications made to proteins after their initial translation from the genetic code.[18] Many proteins are first translated in an inactive or immature state. These proteins must then go through processes in which chemical functionalities are

added or removed before finally reaching their mature, active form. Many PTMs function as a measure of control for cells to determine when and where a protein becomes active and functional.[14, 18]

Because of their role in determining a protein's level of function and location, PTMs are of interest in the study of diseases such as cancer. If a PTM happens too soon, too late, or not at all, the downstream effects can be devastating on the organism. Protein prenylation is a very specific example of a PTM with wide-ranging health effects when it goes awry. When a protein becomes prenylated, the hydrophobicity of that species is increased and the protein typically becomes anchored in the cell membrane, where it mediates a host of protein-protein interactions and cell signaling activity. One specific family of prenylated proteins, the Ras superfamily, features prominently in cancer research due to the emergence of data linking mutations in this superfamily to nearly 30% of known human cancers.[14-18] When Ras protein is successfully prenylated, it is targeted to cellular membranes where it serves it signaling functions. If the Ras protein that is targeted to the membrane is a mutated version, it can result in constitutive signaling that induces the kinds of cell and tumor growth that lead to human cancers.[14-18]

Proteins that may eventually undergo prenylation have some specific structural similarities. Their C-terminus ends in a common "CaaX" motif, often termed the "CaaX box".[11] This refers to the C-terminal sequence of amino acids that signal for prenylation. The C is cysteine, the "a" represents any aliphatic, or open chain non-aromatic, amino acid, and the X can represent any of the amino acids. Proteins displaying this sequence undergo a series of transformations during the process of prenylation. An enzyme, termed a

prenyltransferase, first attaches the prenyl moiety to the cysteine side-chain via a thioether bond.[11-12] Once the prenyl group has been installed, the -aaX sequence is cleaved off by a protease.[11-12] Following the CaaX box cleavage, the newly free C-terminus of the prenyl-modified cysteine residue is methylated, forming a C-terminal prenylated cysteine methyl ester.[11-12] At this stage, the protein may or may not undergo further modifications.

Of particular interest in this chapter is the protease responsible for the cleavage of the -aax sequence following the action of the prenyltransferase enzyme. There are two enzymes responsible for the majority of the -aaX cleavage in humans; Rce1 and Ste24.[11-13, 15] Ste24 is responsible for the -aaX cleavage of a unique class of proteins called lamins, which are largely responsible for providing structure and stability to most eukaryotic cells.[11] In the processing of lamins, Ste24 induces a second proteolysis 15 residues upstream from its initial CaaX cleavage site.[11] If mutations occur in either protein (Ste24 or the lamin) that prevent the second proteolysis from occurring, the immature lamin protein accumulates in the nuclear membrane, inducing a variety of clinical problems, from skin or bone structural abnormalities to diseases as serious as Hutchinson-Gilford progeria.[12-17, 20]

2.3 Rationale

To better understand the mechanisms of Ste24's upstream endoprotease activity, we used Ste24p from the yeast, Saccharomyces cerevisiae. Yeast is well known for its mating strategy that involves two pheromones, **a**-factor and α-factor, that are required for

successful reproduction. Of note in the production of these pheromones, and of particular interest to the study of protein prenylation, is the post-translational processing of **a**-factor. That species is translated as a larger precursor and undergoes several enzymatic modifications leading to its final, farnesylated, mature form. The protease enzyme that is in part responsible for the processing of **a**-factor is Ste24p, the yeast homologue of the afore-mentioned human Ste24.[11] Newly translated a- factor precursor first undergoes farnesylation at its cysteine residue, the CaaX-box cysteine. It is then subjected to its first proteolytic cleavage by the Ste24p, in which the last three amino acids, the -aaX motif, are removed. This leaves an C-terminal cysteine carboxylic acid. This farnesylated C-terminal cysteine then undergoes methylation, forming a methyl ester. At this point, Ste24p completes a second cleavage, 26 amino acids upstream of its first cleavage site. The **a**-factor will undergo one further cleavage by a different enzyme before reaching its active form and being exported.

The Ste24p enzyme's role in **a**-factor's post translational processing is interesting for a variety of reasons. First, the enzyme's ability to cleave two separate sequences at two broadly separated regions on the same peptide indicates an active site sequence specificity that is fairly promiscuous or, potentially, two separate active sites. It is not yet known if the kinetics of the two cleavages differ. Interestingly, the behavior of Ste24p mimics that it's human counterpart, mammalian Ste24. Mammalian Ste24 is responsible for multiple cleavages of its substrate, prelamin A. Incorrect processing of prelamin A has been observed in the onset and progression of such devastating diseases as progeria. Understanding more about the behavior and kinetics of Ste24p could lead to the elucidation

of similar details of the behavior and kinetics of its mammalian counterpart and its related role in human disease.

Previous work by Jeff Vervacke in the Distefano Research group, in conjunction with collaborator Chris Hyrcyna at Purdue, has provided insight into the cleavage kinetics of Ste24p by incorporation of a donor-quencher pair (aminobenzoic acid, Abz and 2,4-dinitrophenol, Dnp) across the upstream cleavage site on **a**-factor.[20] The resulting **a**-factor analogue can be subjected to cleavage by Ste24p and the enzyme's activity can be measured by quantification of the increase in fluorescence intensity upon the liberation of the Abz-modified fragment from the Dnp-modified rest of the peptide.

2.4 Methods

All reagents and solvents were used as received. All the solvents were HPLC grade. DIEA and TFA were of Sequalog/peptide synthesis grade. Standard Fmoc/HCTU chemistry was used for SPPS of peptides.[21-22] Fmoc-based SPPS was performed either manually or using a Protein Technologies PS3 automated peptide synthesizer. Additional steps performed on resin (side-chain deprotection and resin cleavage) were carried out using a polypropylene syringe equipped with a porous polypropylene disc at the bottom. Peptide synthesis and other transformations were performed at room temperature unless otherwise indicated. Peptides were analyzed using reversed-phase high performance liquid chromatography (RP-HPLC) and the products were detected at 220 nm. The analytical column used was a C_{18} Agilent Microsorb-MV 100-5 (4.6 x 250 mm) and the preparative column was a Phenomenex Luna C_{18} (10 μm, 10.00 x 250 mm). The buffer solutions used

for HPLC analysis and purifications were: Buffer A (0.1% TFA in H_2O) and Buffer B (0.1% TFA in CH_3CN). The retention times of the peptides are based on a flow rate of 1 mL/min and on a gradient increase of 1% Buffer B/min after equilibration of the column with 1% Buffer B. The solutions containing purified peptides were lyophilized and analyzed by ESI-mass spectrometry.

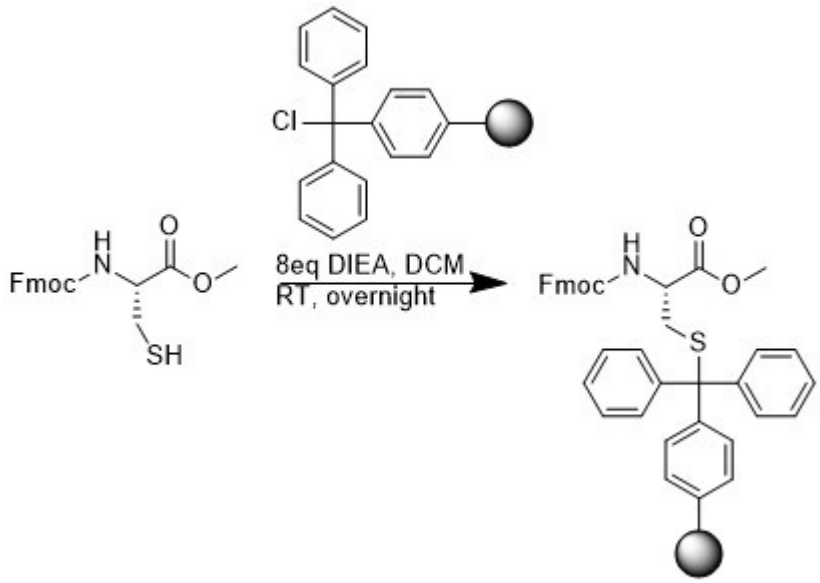
2.4.1 Synthesis of Fmoc-Cys-OCH_3 (2.1)

Fmoc-Cys-OH (0.171 g, 0.5 mmol) was dissolved in CH_3OH (15 mL) in a round bottom flask and 14 drops of concentrated HCl were added to the solution. The reaction was allowed to proceed with stirring at room temperature overnight resulting in the formation of a white, flocculent solid. The entirety of the resulting suspension was then dissolved in acetone and evaporated under vacuum to obtain a white solid (0.162 g, 91% crude yield) that was used without further purification. ^{1}H NMR (DMSO, 400 MHz) δ: 1.38 (t, 1H), 3.03 (m, 2H), 3.83 (s, 3H), 4.25 (t, 1H), 4.63 (m, 2H), 4.69 (m, 1H), 5.70 (d, 1H), 7.33 (dd, 1H), 7.35 (dd, 1H), 7.37 (dd, 1H), 7.41, (dd, 1H), 7.43 (dd, 1H), 7.45 (dd, 1H), 7.63 (d, 1H), 7.78 (dd, 1H). ESI-MS $[M+H]^{+1}$: 357.1, found 357.1.

2.4.2 Synthesis of Fmoc-Cys(Chlorotrityl Resin)-OCH_3 (2.2)

For resin loading, chlorotrityl resin (0.147 g, 0.25 mmol) was swollen in DMF (20 mL) for 30 min. It was then rinsed with CH_2Cl_2 (3 x 2 mL), before compound **2.1** (0.357 g, 4 eq.) dissolved in minimal CH_2Cl_2 was added. DIEA (0.174 mL, 0.129 g, 4 eq.) was then added to the mixture and the reaction was allowed to proceed at room temperature on a rotisserie overnight. After 12 h, CH_3OH (0.05 mL) was added to the reaction mixture to

cap any unreacted chlorotrityl sites on the resin. After 5 min of reaction at room temperature, the resin was washed with DMF (3 x 2 mL), CH_2Cl_2 (3 x 2 mL) and was then allowed to dry in a dessicator overnight. Prior to using the resin for SPPS, it was analyzed to determine the loading of Fmoc-Cys-OCH_3 present. To accomplish that, an Fmoc cleavage test was used.[22] Three aliquots of ~ 25 mg of dried resin were weighed out into 1.5 mL Eppendorf tubes. 1 mL of 20% piperidine in DMF (v/v) was added to each tube followed by brief vortex mixing to produce an even suspension of the resin that was mixed on a rotisserie for 20 min. The tubes were then centrifuged to pellet the resin beads and 10 µL of the supernatant from each tube was added to a new Eppendorf tube and diluted to 1 mL with DMF. The amount of Fmoc protecting group released from these samples was then determined by measuring the absorbance at 301 nm using an extinction coefficient of 3,780 $M^{-1} \cdot cm^{-1}$. This analysis yielded a value of 0.55 meq/g resin that was then used for subsequent SPPS.



Scheme 6: Loading of the resin using the cysteine side-chain anchoring methodology described in Diaz et al.

2.4.3 Synthesis of peptide 1a.

Peptide synthesis was performed manually using Fmoc-Cys(Chlorotrityl Resin)-OCH_3 (**2.2**, 0.181 g, 0.1 mmol) using standard conditions for Fmoc SPPS. In brief, Fmoc deprotection was accomplished by treating the resin with 20% piperidine in DMF (2.5 mL, v/v) for 20 min followed by rinsing with CH_2Cl_2 (2.5 mL) and DMF (2.5 mL). Amide bond formation was accomplished by dissolving the desired Fmoc-protected amino acid (4 equiv) and HCTU (4 equiv) in a solution of N-methyl morpholine in DMF (4 mL, 0.4 M). This was allowed to react briefly until the solution appeared pale yellow in color. The resulting activated amino acid solution was then added to a fritted syringe containing the rinsed, Fmoc-deprotected resin and allowed to react on a rotisserie for 45 min, at which

time the solution was drained and the resin was washed with CH_2Cl_2 (2.5 mL) and DMF (2.5 mL). To ensure complete coupling, a small aliquot of resin was subjected to the Kaiser test for free amines[22]. In cases where incomplete coupling was noted, the coupling reaction was repeated. Following each successful coupling, the new amino acid's N-terminal Fmoc group was removed using a treatment of 20% piperidine in DMF (2.5 mL, v/v) for 20 min, followed by a rinse with CH_2Cl_2 (2.5 mL) and DMF (2.5 mL). The final aminobenzoic acid residue in its Fmoc protected form, was coupled in the same manner as noted above. After the final Fmoc deprotection was completed, the peptide was subjected to global deprotection conditions and resin cleavage using Reagent K which was prepared freshly and consisted of phenol (250 µL), thioanisole (250 µL), 1, 2-ethanedithiol (125 µL), and H_2O (125 µL) diluted to 5 mL with TFA. It is important to note that this cocktail has a strong odor and is severely caustic, so care must be taken when it is used. The described Reagent K cocktail was added to the resin in the syringe and allowed to react on a rotisserie (in a hood, to prevent the release of the stench) for 1.5 to 2 h. The peptide was then precipitated by the addition of $(CH_3CH_2)_2O$ (10-50x the volume of the cleavage cocktail used). The precipitate was recovered by centrifugation, washed with additional $(CH_3CH_2)_2O$ and dried under a stream of N_2 (g). After drying for approximately 30 min, the precipitate was dissolved in a mixture of CH_3CN and H_2O (1:1, v/v) containing, 1% CH_3CO_2H (v/v), flash frozen, and lyophilized to obtain the crude product. Next, the crude material was dissolved in a minimum volume of CH_3CN and H_2O (1:1, v/v) containing 1% CH_3CO_2H (v/v) and purified via semi-preparative reversed-phase HPLC using a C_{18} Agilent Microsorb-MV 100-5 (4.6 x 250 mm) column. For each run, 2.5 to 3 mL of crude

peptide solution was injected onto the column which was eluted with a gradient of mobile phases consisting of Buffer A (H_2O containing 0.1% TFA, v/v) and Buffer B (CH_3CN containing 0.1% TFA, v/v) using a flow rate of 2 mL/min and monitoring the absorbance at 220 nm. The elution profile consisted of a gradient that began in 10% buffer B that increased to 25% B in 10 min, and then to 40% B in 50 min. The material began to elute almost immediately after 10 min as evidenced by the bright yellow color of the eluent, due to the peptide's dinitrophenyl chromophore. Fractions from this purification were analyzed by ESI-MS and the fractions containing the appropriate mass were analyzed individually by analytical HPLC using a similar gradient to that used in the purification. The fractions of highest purity (as determined by analytical HPLC) were combined and lyophilized yielding 25 mg (6.3% yield, 90% purity) of purified peptide. HPLC R_T: 27 min. MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{176}H_{267}N_{43}O_{56}S_2^{4+}$: 985.9705; found 986.45. **(S 12, S 18)**

2.4.4 Synthesis of 1b—Farnesylated L-Cys-Methyl Ester

Peptide **1a** (10 mg, 0.002 mmol, 1 equiv) was dissolved in a minimum volume of DMF (1-2 mL). To this solution was added $Zn(OAc)_2 \bullet 2H_20$ (10x the mass of the peptide) dissolved in a minimum volume of HPLC buffer A (1 mL, 0.1% TFA in H_2O). Farnesyl bromide (10 equiv) was then added to the peptide solution. The reaction mixture often became turbid under these conditions as the farnesyl bromide precipitated. The reaction was allowed to proceed overnight with stirring and monitored by analytical HPLC. Next, it was centrifuged to remove any precipitated farnesyl bromide and then injected directly on to a semi-preparative C18 HPLC column for purification. The column used was a C_{18}

Agilent Microsorb-MV 100-5 (4.6 x 250 mm). The elution profile consisted of a gradient that began in 10% buffer B that increased to 25% B in 10 min, and then to 100% B in 70 minutes. Fractions containing **1b** were pooled and lyophilized to yield a yellow solid (1.2 mg, 14.4% yield). HPLC R_T: 54 min. **(S 1)** MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{190}H_{290}N_{44}O_{56}S_2^{4+}$ 1037.5171; found 1037.5172.

2.4.5 Synthesis of 2a—D-Cys-Methyl Ester

This peptide was prepared using the method described for **1a**. Starting with Fmoc-D-Cys(Chlorotrityl Resin)-OCH_3 (2.2 0.181 g, 0.1mmol), 9.85 mg of 2a was obtained (2.5 % yield). HPLC R_T: 27 min. MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{176}H_{267}N_{43}O_{56}S_2^{4+}$: 985.9705; found 985.9. **(S 18, S 19)**

2.4.6 Synthesis of 2b—Farnesylated D-Cys-Methyl Ester

Peptide **2b** was prepared as described for **1b**. Starting from **2a** (5 mg, 0.00l mmol), no pure material was obtained.

2.4.7 Synthesis of 3a—L-Cys-acid

This peptide was prepared using the same method described for **1a** but performed using standard Wang resin to obtain the desired C-terminal acid. Starting with Fmoc-Cys(Trt)-O-Wang Resin (1 g, 1.2 mmol), 124 mg of **3a** was obtained (2.6 % yield). HPLC R_T: 21 min. MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{175}H_{265}N_{43}O_{56}S_2^{4+}$ 982.4666; found 982.5.

2.4.8 Synthesis of 3b—Farnesylated L-Cys-acid

Peptide **3b** was prepared as described for **1b**. Starting from **3a** (25 mg, 0.006 mmol), 1.5 mg (6% yield, 90 % purity) of **3b** was obtained. HPLC R_T: 49 min. MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{189}H_{288}N_{44}O_{56}S_2^{4+}$ 1034.0132; Found 1034.0131. **(S 13, S 14)**

2.4.9 Synthesis of 4a—L-Cys-amide

This peptide was prepared using the same method described for **1a** but performed using standard Rink amide resin to obtain the desired C-terminal amide. Starting with Fmoc-Cys(Trt)-amide Resin (0.434 g, 0.2 mmol), 33.4 mg of **4a** was obtained (4.4 % yield). HPLC R_T: 20 min. MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{175}H_{266}N_{44}O_{55}S_2^{4+}$ 982.2206; Found 982.2.

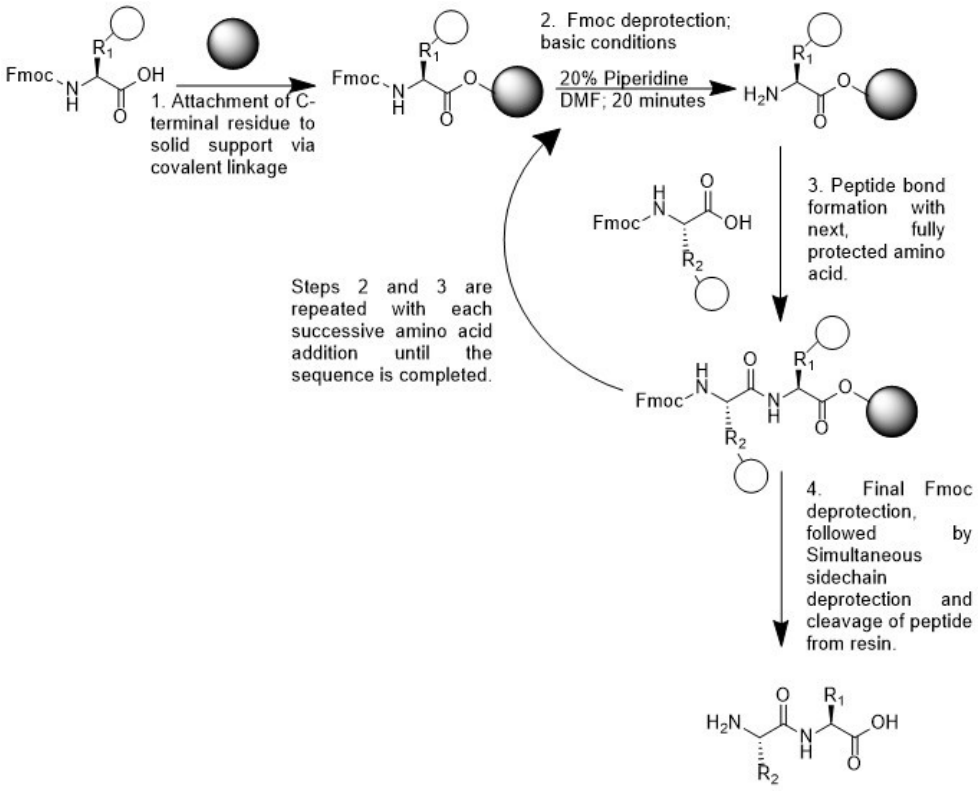
2.4.10 Synthesis of 4b—Farnesylated L-Cys-amide

Peptide **4b** was prepared as described for **1b**. Starting from **4a** (10 mg, 0.002 mmol), 0.63 mg (6.3 % yield, 91 % purity) of **3b** was obtained. HPLC R_T: 55 min. MS (ESI-TOF) m/z: $[M+4H]^{4+}$ Calcd for $C_{189}H_{289}N_{45}O_{55}S_2^{4+}$ 1033.7672; Found 1033.7672. **(S 15)**

2.4.11 Ste24p Assay

The synthesized internally quenched cleavage probe peptides were sent to our coallaborators at Purdue University. There, graduate student Erh-Ting Hsu in the Hrycyna lab performed the Ste24p fluorescence-based assays described here. Membranes containing 10 or 40 μg of active Ste24p enzyme were mixed with the probes (30 μM) in a 96-well plate along with 100mM Tris-HCl buffer at pH 7.5 and a cocktail of protease inhibitors (10 μg/μl chymostatin, 10 μg/μl leupeptin, 1% aprotinin and 2 mM AEBSF). Fluorescence was measured at 420 nm at 1-minute intervals for 30 minutes at 30°C using

a Bio-Tek Synergy H4 fluorometer. The collected data were exported and initial linear slopes were graphed using Microsoft Excel.



Scheme 7: General strategy for Fmoc-Solid Phase Peptide Synthesis

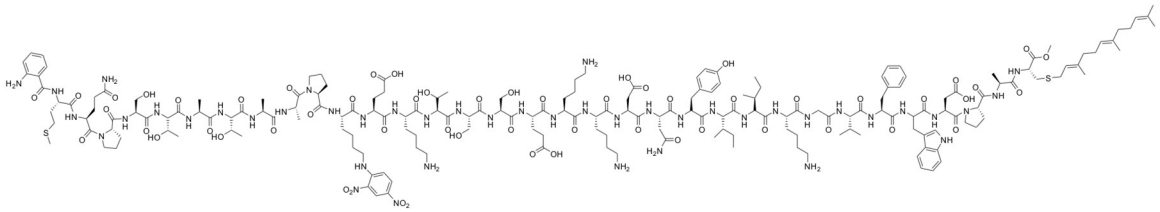


Figure 4: Compound **1b**

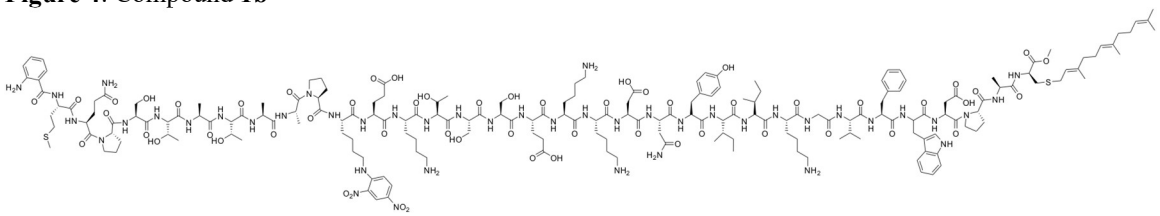


Figure 5: Compound 2b

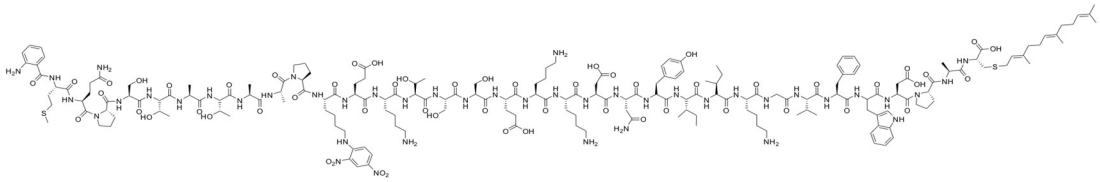


Figure 6: Compound **3b**

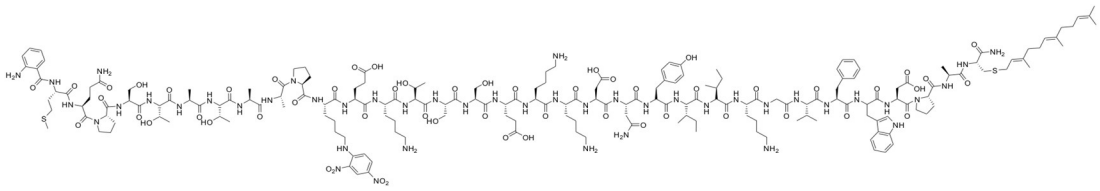


Figure 7: Compound **4b**

2.5 Results and Discussion

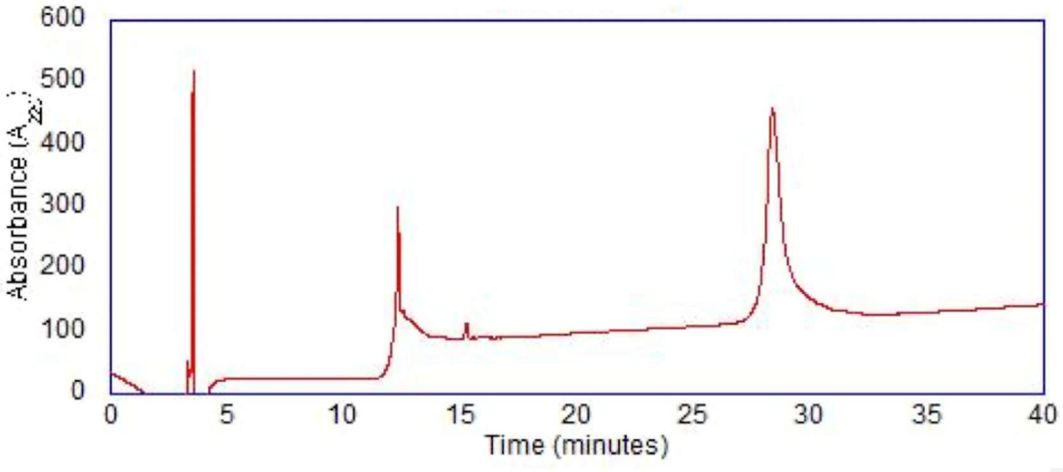
In Table 3.1, the specific activities of the four synthesized internally quenched probes are shown. While a slight increase in specific activity is observed in for compound 3b, there is not sufficient evidence to support that the increase is significantly different from the specific activities of the other analogues. (**S 16, S 17**)

Substrate (30 µM)	**10 µg Ste24p** Specific activity (pmol/mg/min)	**40µg Ste24p** Specific activity (pmol/mg/min)
Compound **1b**	298	259
Compound **2b**		
Compound **3b**	382	412
Compound **4b**	356	366

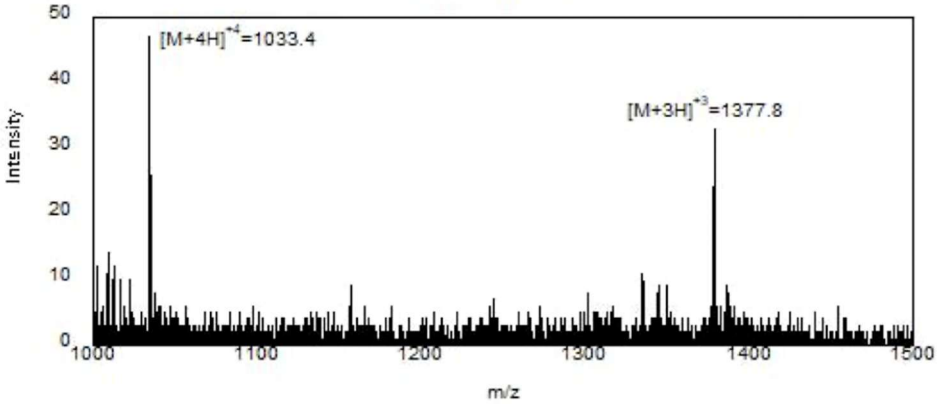
Table 1: Ste24p cleavage kinetics in response to changes in C-terminal modifications of synthetic a-factor peptide analogue.

These data indicate that the C-terminal functionality is not critical for the N-terminal cleavage completed by the Ste24p enzyme. On a relatively small scale, this indicates that the additional synthetic steps required to produce 'biologically accurate" C-terminal methyl ester analogues may not be necessary. It is known that the C-terminal methyl ester is required for complete maturation of the yeast mating hormone a-factor, but it is unknown how critical this transformation is in the processing of lamin proteins in mammals. These data are supported by previously published results indicating that mutation of yeast to remove Ste14 activity, and thus methyl transferase activity, do not show marked impairment in the function of several proteins known to be methylated under normal conditions.[26] Taken together, this indicates the potential for a synthetic "shortcut" in the production of probes to further study the activity of Ste24p. It also suggests that other dual endoproteases, in particular the human counterpart to Ste24p, Ste24, might tolerate a broader range of C-terminal functionalities than previously thought.

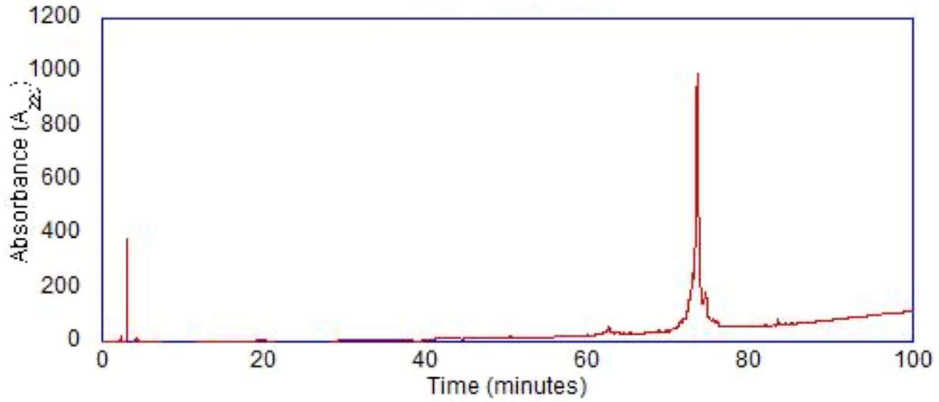
2.6 Supporting Information



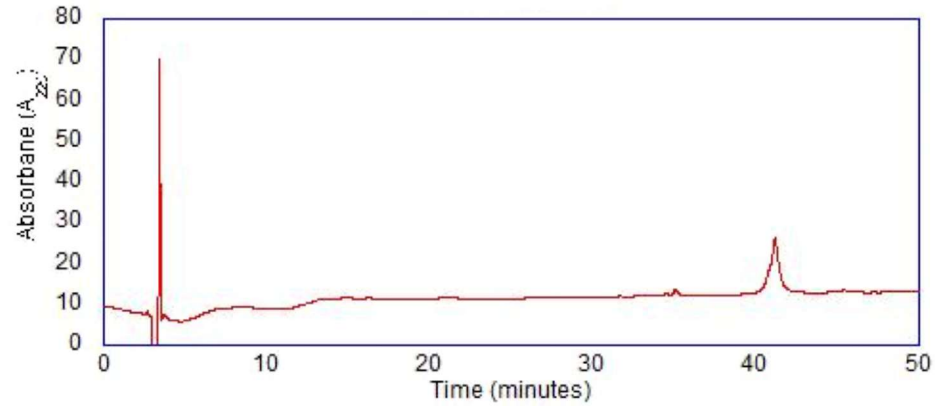
S 12 C-18 analytical chromatogram of **1a**. Injections of 50 µL of each sample were completed. Data shown were collected at 220nm, with a gradient of 0-20% B in 10 min, followed by 20-60% B at a 1%/min gradient.



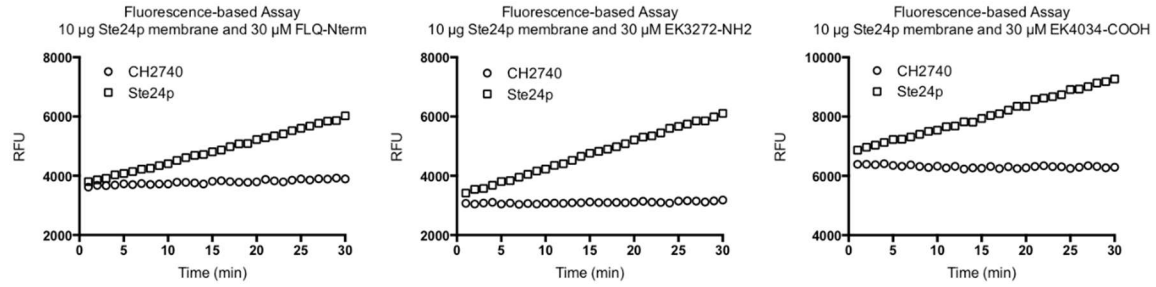
S 13 ESI-MS, positive mode for **3b**. Compound **3b** was dissolved in a 1:1 acetonitrile:water, 1% acetic acid solution and 100 µl of this solution was injected



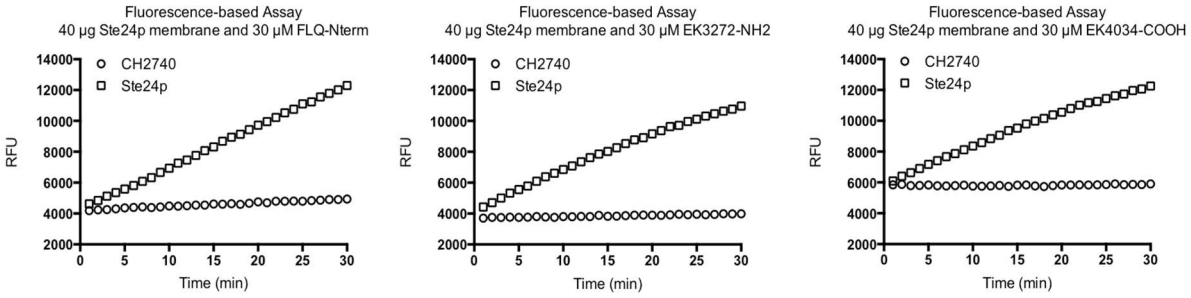
S 14 HPLC C-18 analytical trace for **3b**. Injections of 50 µL of each sample were completed. Data shown were collected at 220nm, with a gradient of 0-100% B at a 1%/min gradient



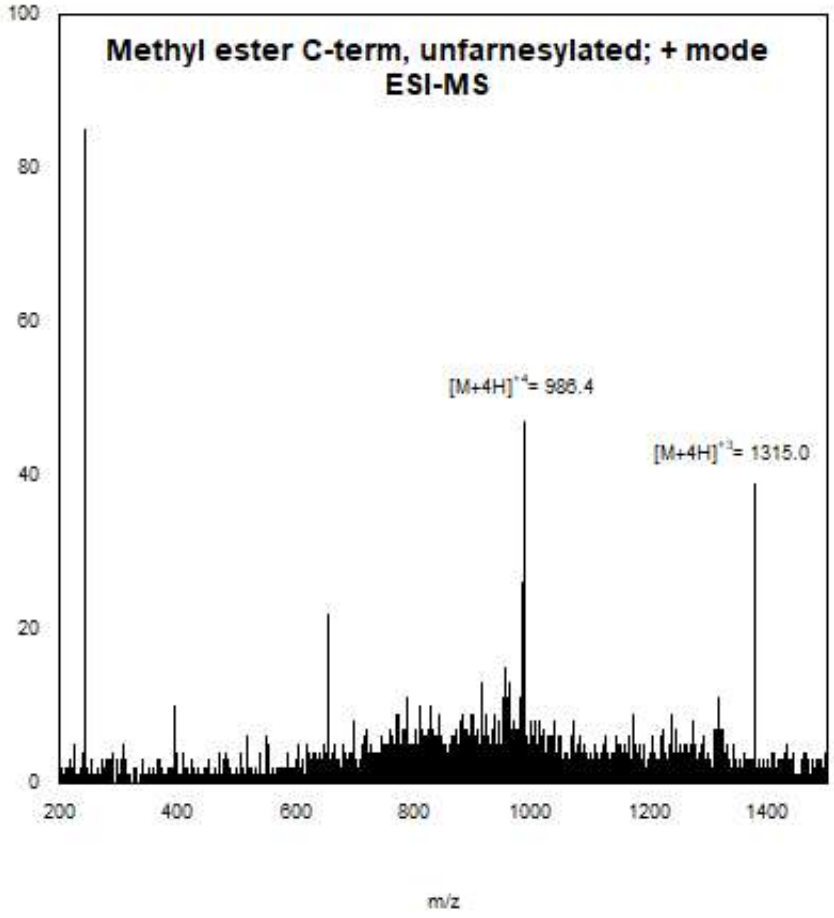
S 15 HPLC C-18 analytical trace for **4b**. Injection of 50 µL of each sample were completed. Data shown were collected at 220nm, with a gradient of 0-20% B in 10 min , followed by 20-60% B at a 1%/min gradient.



S 16 Ste24p fluorescence assay with 40ug Ste24p enzyme. Data Collected by Erh-Ting Hsu, Purdue University



S 17:Ste24p fluorescence assay with 40ug Ste24p enzyme. Data Collected by Erh-Ting Hsu, Purdue University



S 18 Representative ESI-TOF data for purified L and D Methyl Ester Peptides (1a and 2a). Compound was dissolved in a 1:1 acetonitrile:water, 1% acetic acid solution and 100 µl of this solution was injected

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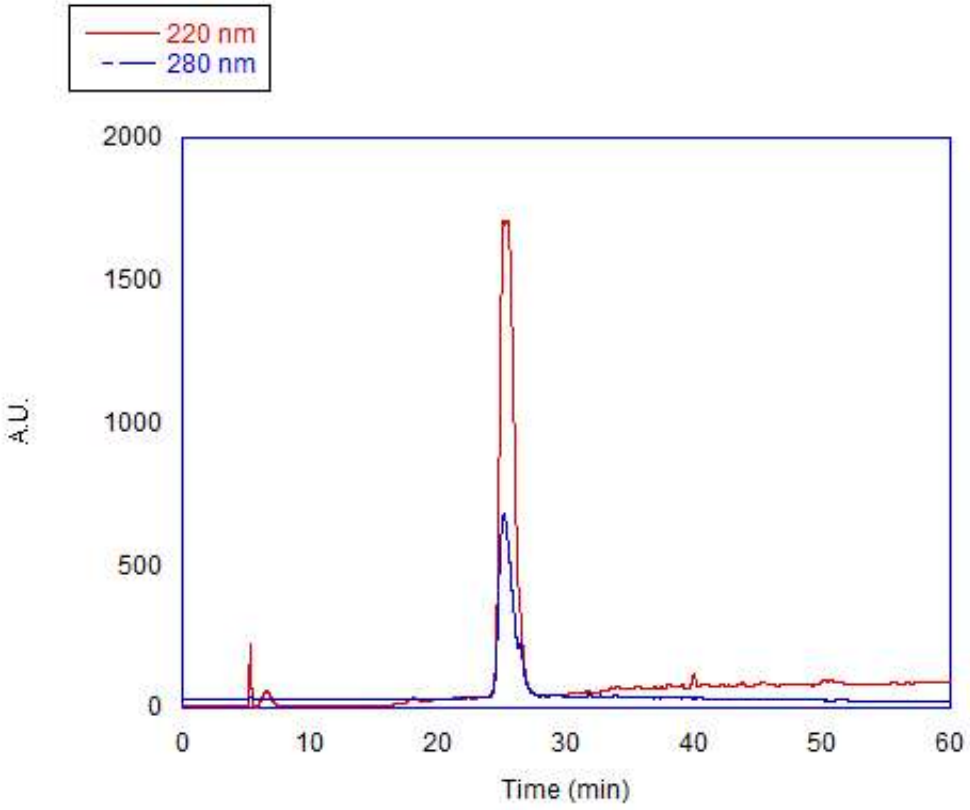

S 19 : : C-18 Analytical Trace of Purified Peptide **2a**. An injection of 50 µL of each sample was completed. Data shown were collected at 220 nm and 280 nm, with a gradient of 5-100% B at a 2%/ min gradient

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