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Eddy Pineda 04/03/2023

Aldehyde and CyClick Cyclization Impacts on Peptide Cellular Permeability

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Aldehyde and CyClick Cyclization Impacts on Peptide Cellular Permeability
by Eddy A. Pineda

Peptides and other hydrophobic molecules are considered viable candidates for drug discovery and biotherapeutic development due to their high affinity and specificity, but they suffer from poor cell penetration and stability. One potential solution to these issues has been the cyclization of peptides, but they also suffer from poor cellular permeability. A cyclization method known as CyClick has been developed in recent years that seek to overcome these issues. This method results in forming a 4-imidazolidinone moiety which introduces a ring to the peptide. A peptide's ability to permeate the cell is due to varying structural motifs or conformations such as cyclization or the presence of certain functional groups. This study seeks to determine the effect of 4-imidazolidinone and aldehydes on peptide cell permeability. A series of cyclic peptides, peptide aldehydes, and CyClick peptides were synthesized and tagged with a chloroalkane tag to examine permeability. Using a recently developed chloroalkane penetration assay (CAPA), the permeability of these peptides was examined in cell models. The presence of both 4-imidazolidinone and aldehydes increased the permeability of peptides compared to cyclic and linear peptides not containing these structures. Further studies are being done to examine the impact of multiple aldehydes and their presence on cyclic peptides on cellular permeability. If these structures increase cellular permeability at a comparable rate to other permeability methods, then peptides aldehydes and CyClick-made aldehydes can be explored for future use in therapeutics and drug development.

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Table of Contents

Chapter 1: Introduction *1*

Peptides as Drug Candidates **1**

CyClick Chemistry and Purpose **3**

Chapter 2: Materials and Methods *6*

Synthesis of Linear Peptides **6**

Synthesis of Fmoc-Alanine-Aldehyde **7**

Synthesis of Aldehyde Resin **7**

Synthesis of Aldehyde Linear Peptides **8**

Synthesis of Macrocyclic Peptides **9**

Synthesis of CyClick Peptides **9**

Synthesis of Aldehyde Macrocyclic Peptides **10**

Synthesis of Chloroalkane Tag **11**

Deprotection of O-Allyl Glutamic Acid Containing Peptides **12**

Coupling of Chloroalkane Tag and Peptide **12**

Chloroalkane Penetration Assay **12**

Chapter 3: Results and Discussion *14*

Chapter 4: Conclusions and Future Directions *22*

References *23*

Supplemental Information *24*

Chapter 1: Introduction

Peptides as Drug Candidates

Peptides are a type of macromolecule formed through peptide bonds between amino acids which serves as the building block for the secondary structure of peptides. Peptides have been studied extensively for their use as a therapeutic as they are implicated in a variety of biological functions such as protein-protein interactions, treating disease, and reducing inflammation.[1, 2] Historically, therapeutic peptides have acted as hormones, growth factors, and ligands since the 1920s with the development of insulin, a 36 amino acid peptide.[3-5] Since then, there have been a long list of FDA approved peptide drugs developed such as Exenatide, used to treat type two diabetes, and Degarelix, used to lower testosterone levels in prostate cancer patients (Figure 1A and 1B).[4] Peptides such as those tend to be linear peptides, but there are other FDA-approved peptides used such as Pasireotide, used to treat Cushing's disease, which are cyclic (Figure 1C).[4]

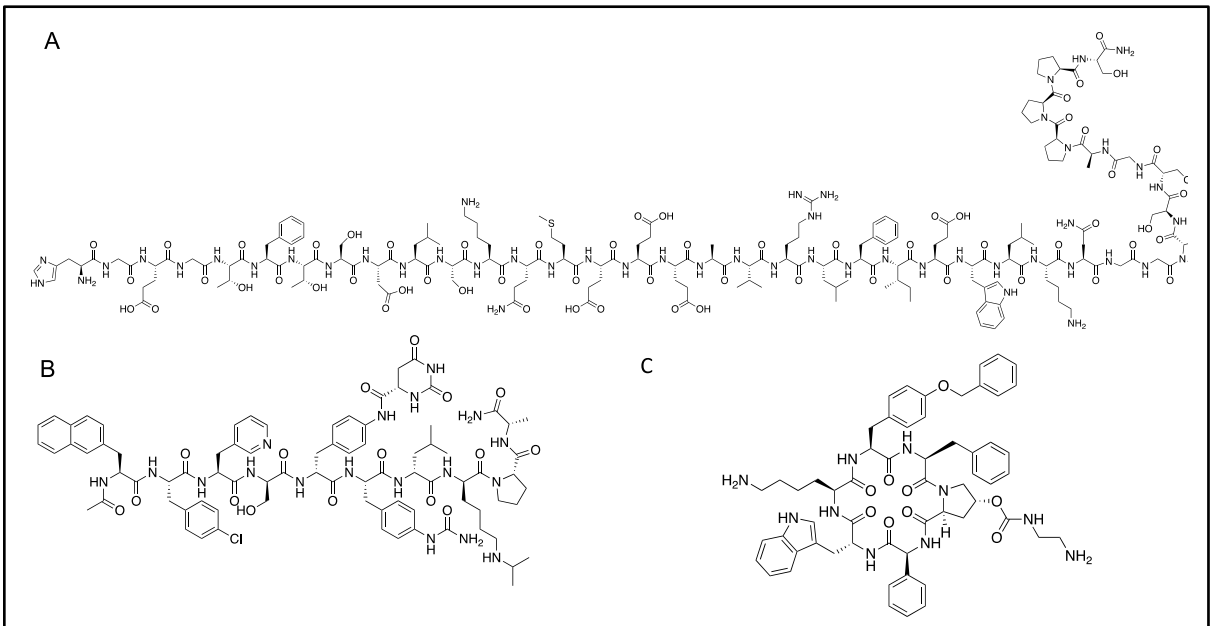


Figure 1. (A) Structure of Exenatide. (B) Structure of Degarelix. (C) Structure of Pasireotide

A majority of the peptides observed as drug candidates or in nature usually fall under two different classes: linear and cyclic.[3] The class they are in factors significantly into their pharmacokinetic properties with some peptides being better than others including small molecule drugs.[3] Factors that impact the ability of a peptide as a potential therapeutic include binding affinity, selectivity, and specificity to a desired target, a high ability to permeate cell membranes, and plasma stability.[3] Despite their versatility in the body, linear peptides have been considered poor drug candidates with low therapeutic properties.[3] They suffer from multiple shortcomings which hinder their viability to serve as drugs. Structurally, they are not stable molecules inside the body or cell as they can be degraded inside the body by various proteolytic enzymes.[3] The lack of stability stems from the lack of stabilizing interactions present in the secondary and tertiary structures of peptides such as hydrogen bonding, ionic interactions, covalent bonds, and other non-covalent bonds.[3, 4] They have low cellular permeability as their length and structure impact their ability to cross the cell membrane to reach their target.[4]

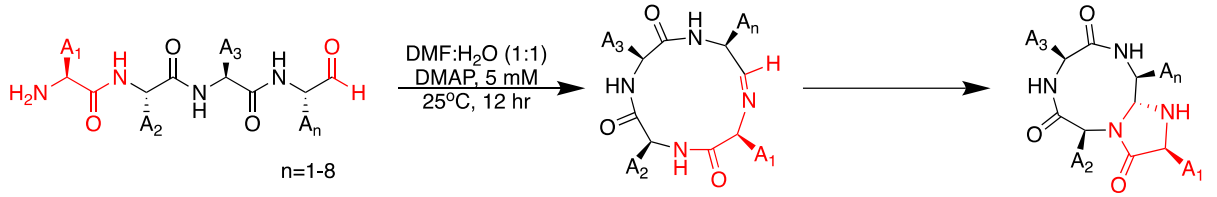
To alleviate some of the issues plaguing linear peptides, scientists have attempted to incorporate modifications into peptides at various points during their synthesis. They have done N-methylation of the amine nitrogen as it decreases the energy difference between the *cis*- and *trans*- conformations of the peptide bonds.[3] The process also eliminates free amine groups thus limiting the impact of desolvation on the energetics of the system.[3] N-methylation increases cellular permeability, but it is difficult to do further modifications on them.[3] Modifying specific conformations of the peptide such as α-alkylation, stabilize the structure of the peptide, increase cellular permeability, but reduces solubility.[3] The use of non-canonical amino acids and D-amino acids as opposed to L-amino acids during synthesis stabilized the structure of the peptide, making it resistant to proteolysis.[4, 5]

The most consequential modification involves the cyclization of peptides. Many peptides found in nature are cyclic, and they have been studied extensively to determine their properties. Cyclic peptides are known to be more stable, cell-permeable, potent, and can target areas considered as undruggable such as protein-protein interactions.[3, 6, 7] As for their stability, cyclization removes the presence of terminal ends on the peptide which makes it difficult for a protease to degrade the peptide.[8] They are also more permeable into the cell due to their rigid secondary structure that ensures less hydrogen bonding occurs between the surrounding environment and the amide groups on the peptide. Less hydrogen bonding means there is a lower entropic penalty to transportation across the cell membrane where those bonds would have to be broken.[6]

The advent of macrocyclic peptides has spurred further interest in them and their ability as drug candidates. These studies have concluded that cyclic peptides have their limitations and suffer from setbacks which limit their ability to be effective therapeutics. One issue pertains to their synthesis as these peptides need to be synthesized on-resin or in low concentrations when in solution. If these conditions are not met, then various side reactions can occur where the peptide can oligomerize or dimerize.[9] Another issue is the cellular uptake of these macrocyclic peptides and what makes be transported into the cell with higher efficiency. In general, cyclization increases cell permeability, but this is not true for all macrocyclic peptides.[8] Not every peptide triggers an event of endocytosis or entry as the properties that increase the permeability of a peptide are not well-characterized. In addition, macrocyclic peptides cannot be modified to increase their permeability to be considered as drug candidates for targets located inside of a cell.[6]

CyClick Chemistry and Purpose

To overcome the synthesis issue with cyclization, the Raj lab developed a new pathway for the synthesis of cyclic peptides known as CyClick Chemistry. Using peptide aldehydes, a cyclic imine is made between the N-terminus of the peptide and the C-terminal aldehyde (Scheme 1).[9] A subsequent nucleophilic attack between the amine in red and the imine to synthesize a cyclic peptide containing a 4-imidazolidinone moiety if synthesized. CyClick has proved useful to synthesize cyclic peptides due to its high stereoselectivity, chemoselectivity between the N-terminus and aldehyde, application to varying ring sizes, stability, and reaction kinetics.[9] There is



Scheme 1. Linear peptide undergoes an intramolecular reaction to form a cyclic imine intermediate before forming the final product containing 4-imidazolinone.

also no need for any coupling agents, metals, or any external compounds to promote cyclization.[9] The reaction can be done on peptides containing both canonical and non-canonical peptides, resulting in a stable peptide.

The formation of the 4-imidazolinone motif is of note as the structure is found in many pharmaceutical and other biologically active compounds.[9] An example of such a molecule is ML298 which is a known inhibitor of Phospholipase D2 (Figure 2).[9] The feature is also known to improve the pharmokinetic properties of molecules while maintaining their biological activity.[10] Due to its presence on the CyClick peptides, there is a potential for possible drug development using them. The first hurdle in proving their feasibility as therapeutic agents would be determining their cell

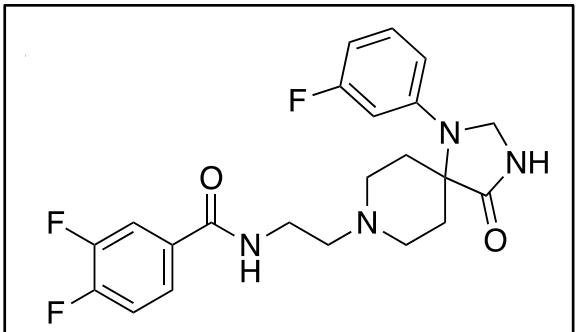


Figure 2. Structure of ML298.

permeability and how it compares to other types of peptides containing groups that impact their permeability.

Throughout the following thesis, I will examine the impact of the 4-imiziladinone moiety and peptide aldehydes on the permeability of peptides to determine their functionality as potential drug candidates. To achieve this, I will examine linear peptide aldehydes and simple linear peptides of varying lengths to determine the impact of aldehydes on permeability. I will also examine CyClick peptides, their regular macrocyclic counterparts, and macrocycles containing an aldehyde to determine both if CyClick and aldehyde synthesized macrocycle peptides improve permeability. To gauge levels of permeability, we will be working with a collaborator from Tufts University, Dr. Joshua Kritzer, who has developed one of few assays that can quantitatively measure the permeability of a molecule. This method is known as a chloroalkane penetration assay (CAPA) which is a high-throughput pulse-chase fluorescence method (Figure 3).[11] Using HaloTag technology, cells are incubated with molecules containing a chloroalkane tag which binds to the HaloTag protein. After a subsequent wash, they are incubated with a chloroalkane-tagged dye which binds to free HaloTag protein. The cells are analyzed by flow cytometry in 96 well plates containing 10,000 cells per sample. Normalization of the fluorescence

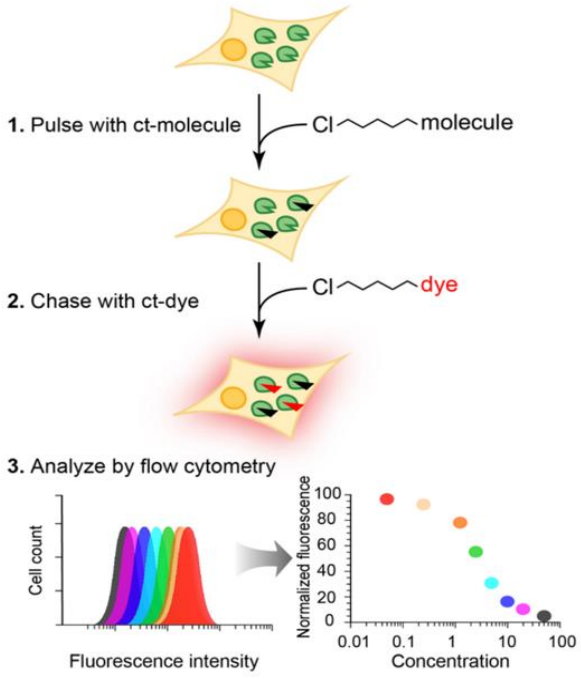


Figure 3. Chloroalkane Penetration Assay (CAPA) involves pulsing the cell with a chloroalkane tagged molecule followed by a chase with a chloroalkane tagged dye before being analyzed.[11]

intensity gives rise to before being used to determine their CP_{50}, the concentration of peptide at which 50% of peptide has permeated the cell membrane and is bound to the HaloTag protein.[11]

Chapter 2: Materials and Methods

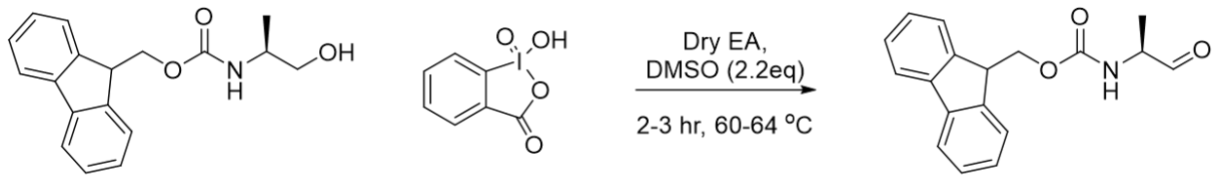
Synthesis of Linear Peptides

Various linear peptides were synthesized from the N to C terminus through solid-phase peptide synthesis using a variety of canonical Fmoc-protected amino acids. For manually synthesized peptides, the process began weighing out one equivalent CreoSalus™ Rink Resin onto a solid phase extraction tube. The resin was allowed to swell for 30 minutes in DMF before deprotection using 20% Piperidine in DMF. The deprotection reaction was allowed to shake on a Burell™ Model 75 Wrist-Action Shaker for approximately ten minutes before being washed twice with DMF, Methanol, and DCM. Ending the process with a third DMF wash to begin coupling of the Fmoc-protected amino acids. The process of amino acid coupling involved weighing out four equivalents of both Fmoc-protected amino acid and activator base, 1-hydroxy-7-azabenzotriazole (HOAt), onto a 15 mL falcon tube using an analytical scale. The mixture was dissolved in DMF, and four equivalents of activator, N, N'-Diisopropylcarbodiimide (DIC), were added. The resulting solution was poured onto the solid phase extraction tube before being placed on the shaker for approximately 25 minutes. Washing and coupling steps were repeated until the desired peptide was synthesized. Some linear peptides were synthesized using a Liberty Blue™ peptide synthesizer with an amino acid concentration of 0.20 M. The deprotection solution was piperidine, the activator was DIC, and the activator base was Oxyma. To cleave the peptide from the rink resin, a solution of 95% trifluoracetic acid (TFA), 2.5% H_2O, and 2.5% triisopropylsilane (TIS) was added to the solid phase extraction tube before being placed on the shaker for approximately 1-2 hours. The peptide was filtered into a scintillation vial before being placed under air to remove excess

TFA. A waxy, solid was formed which was then dissolved in 50% ACN and 50% H_2O to begin the purification process. Peptides before purification and analysis were stored in a desiccator. Synthesized peptides were analyzed and purified with MS and HPLC. Fractions were collected, added together into a 15 mL falcon tube, and lyophilized before being stored in the cold room.

Synthesis of Fmoc-Alanine-Aldehyde

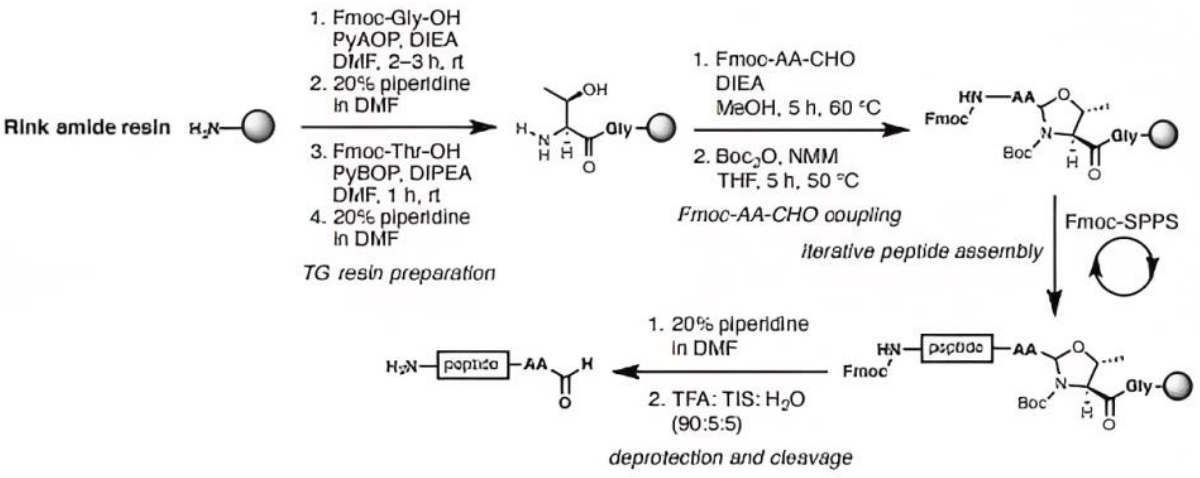
A round bottom flask with three necks was wrapped in aluminum and purged using N_2 gas. Once the system was purged, 1 equivalent of L-alaninol was added to the flask followed by dry ethyl acetate (10 mL/mmol L-alaninol). 1.5 equivalents of 2-iodoxybenzoic acid (IBX) and 2.2 equivalents of DMSO were then added relative to the L-alaninol. The reaction mixture was refluxed under dark and inert conditions for three hours at around 60°C-64°C (Scheme 2). The product underwent a workup where IBX and other side-products were filtered off using ethyl acetate and deionized water. This process was done three times then tried using Na2SO4 and filtered into a pre-weighed round bottom flask. Afterward, the product was purified using a silica gel column using a hexanes/diethyl ether gradient beginning with 100% hexanes:0% diethyl ether going to 60% hexanes:40% diethyl ether. Fractions containing aldehyde as proved through TLC were collected and excess diethyl ether was removed using a rotovap with the product being a white, flaky powder. The product was stored in the freezer until used for aldehyde resin synthesis.



Scheme 2. Fmoc-alaninol reacts with IBX to form Fmoc-Ala-Aldehyde

Synthesis of Aldehyde Resin

The same method used to synthesize linear peptides manually was used to make a Gly-Thr dipeptide attached to rink resin. After the peptide was synthesized, the dipeptide was deprotected using 20% piperidine in DMF (Scheme 3). In a round bottom flask, one equivalent of deprotected peptide was added, in addition to four equivalents of Fmoc-Ala-Aldehyde and N,N-diisopropylethylamine (DIEA; 100 µL/gram) in methanol (10 mL/g). The reaction mixture was refluxed at 60°C at the lowest spin level for five hours (Scheme 3). Once five hours have passed, the product was washed using a sequence of DMF, methanol, and DCM, before setting up the Boc protection of the aldehyde resin. Boc protection of the aldehyde resin involves adding the resin to a round bottom flask, in addition to five equivalents of 4-methylmorpholine and di-tert-butyl pyrocarbonate (Boc_2O) dissolved in THF (10 mL/g). The reaction mixture was refluxed at 50°C at the lowest spin level for five hours (Scheme 3). The protected aldehyde resin was stored in the desiccator for use in peptide synthesis experiments.



Scheme 3. Rink resin undergoes peptide synthesis using two amino acids before being reacted with Fmoc-Ala-Aldehyde and Boc protected to allow for solid phase peptide synthesis.

Synthesis of Aldehyde Linear Peptides

Various linear peptides were synthesized through solid-phase peptide synthesis using a variety of canonical Fmoc-protected amino acids. For manually synthesized peptides, the process began

weighing out synthesized aldehyde resin. The normal procedure for the synthesis of linear peptides was used (Scheme 3). Other linear peptides were synthesized using a Liberty Blue™ peptide synthesizer with an amino acid concentration of 0.20 M. Synthesized peptides were analyzed and purified with MS and HPLC. Fractions were collected, added together into a 15 mL falcon tube, and lyophilized before being stored in the cold room.

Synthesis of Macrocyclic Peptides

Linear peptides were manually synthesized using 2-chlorotrityl chloride resin. A displacement reaction is done on the resin using DIEA in DMF to create an ester. Afterward, normal solid-phase peptide synthesis steps were taken to synthesize the peptide of choice which then underwent an O-Allyl deprotection on the glutamic acid residue. The amine version of the chloroalkane tag was coupled to the peptide which was then analyzed and purified with mass spectrometry (MS) and HPLC. The purified linear peptide underwent an amide cyclization reaction where one equivalent of peptide was placed in a scintillation vial. Relative to the peptide, four equivalents of HOAt, DIC, and DIEA were all added to the scintillation vial before being dissolved in DMF (10 mL/0.1 mmol). The reaction was allowed to stir at room temperature for 16 hours before being placed inside the Speedvac for 2 hours at 65°C to remove excess solvent. Analysis and purification followed with HPLC and mass spectrometry. Fractions were collected, added together into a 15 mL falcon tube, and lyophilized before being stored in the cold room.

Synthesis of CyClick Peptides

The same method used to synthesize linear peptides manually was used to make various linear peptides with the aldehyde resin. The linear peptides underwent an O-Allyl deprotection on the glutamic acid residue to allow for coupling of the amine version of the chloroalkane tag. The peptide was purified and analyzed with MS and HPLC before being cyclized. Cyclization involved

adding one equivalent of peptide and 10 equivalents of DIEA (relative to the peptide) in DMF (100 mL/0.5 mmol). The peptide was purified and analyzed with MS and HPLC. To cleave the peptide from the aldehyde resin, a solution of 95% TFA, 2.5% H_2O, and 2.5% triisopropylsilane was added to the solid phase extraction tube before being placed on the shaker for approximately 1-2 hours. The peptide was filtered into a scintillation vial before being placed under air to remove excess TFA. A waxy, solid was formed which were then dissolved ACN and H_2O. Peptides before purification and analysis were stored in a desiccator. Synthesized peptides were analyzed and purified with MS and HPLC. Fractions were collected, added together into a 15 mL falcon tube, and lyophilized before being stored in the cold room (Scheme 1).

Synthesis of Aldehyde Macrocyclic Peptides

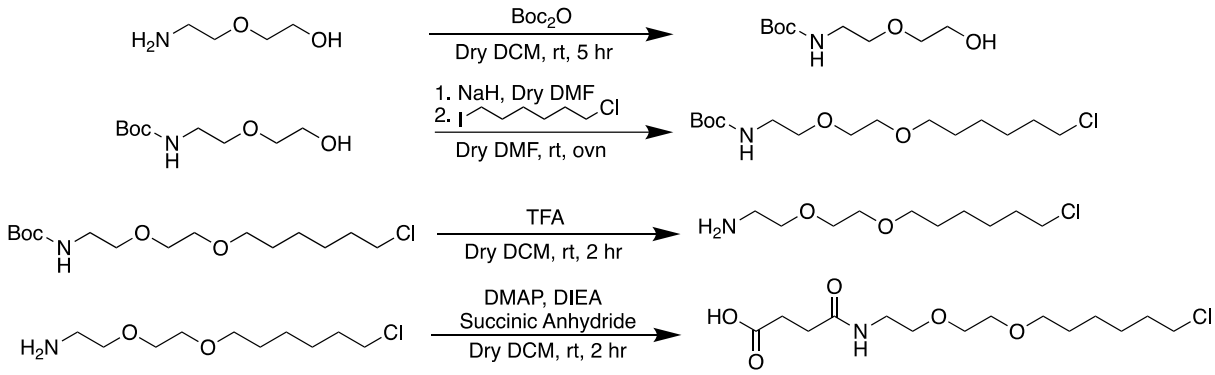
Linear peptides were synthesized using the Liberty Blue™ peptide synthesizer. Peptides were made with the inclusion of a C-terminal O-Allyl protecting group on the glutamic acid residue and a lysine derivative known as dimethyl-lysine (KMe_2). The group was deprotected to allow for coupling of the carboxylic acid version of the chloroalkane tag. Analysis and purification of the peptide were done with mass spectrometry (MS) and HPLC. The purified linear peptide underwent an amide cyclization reaction where one equivalent of peptide was placed in a scintillation vial. Relative to the peptide, four equivalents of HOAt, DIC, and DIEA were all added to the scintillation vial before being dissolved in DMF (10 mL/0.1 mmol). The reaction was allowed to stir at room temperature for 16 hours before being placed inside the Speedvac for 2 hours at 65°C to remove excess solvent. Analysis and purification followed with HPLC and mass spectrometry. Afterward, an aldehyde was inserted chemically using the KMe_2 amino acid. This process was done off-resin. The off-resin process involved using one equivalent or the entirety of the purified peptide, two equivalents of Selectfluor™, and 10 equivalents of pyridine. The peptide was

analyzed and purified with MS and HPLC. Fractions were collected, added together into a 15 mL falcon tube, and lyophilized before being stored in the cold room.

Synthesis of Chloroalkane Tag

20 mmol of 2-(2aminoethoxy)ethanol was placed in a purged round bottom flask along with 50 mL of dry DCM. The flask was placed in an ice bath and allowed to spin as 20 mmol of Boc_2O was added to the mixture. The reaction was left at room temperature for two hours then diluted with DCM and washed with deionized water and brine. The organic layer was collected and dried with sodium sulfate before being placed in a pre-weighed round bottom flask. The solution was concentrated using a rotovap to obtain a colorless liquid. The round bottom flask was purged and the product from the first reaction (2 g, 9.7 mmol) was dissolved in 50 mL of dry DMF. Afterward, 13.44 mmol of NaH was added to the flask as it was placed in an ice bath and left to stir for 30 minutes. After 30 minutes, 24.7 mmol of 1-chloro-6-iodohexane was added to the mixture and allowed to react overnight. Quenching of the second product was done with 1 M HCl and extracted using ethyl acetate. The product was washed with deionized water and ethyl acetate with the organic layer being collected and dried using sodium sulfate before purification. Column chromatography was done using a gradient of 20:80 ethyl acetate/hexanes to 40:60 ethyl acetate/hexanes. The fractions were collected and concentrated using a rotovap to obtain a yellow liquid. The product of the second reaction was dissolved in 40 mL dry DCM and 10 mL TFA as it was in an ice bath before being transferred to room temperature to react for two hours. The resulting product was dissolved in methanol and treated with K_2CO_3 until the reaction was neutralized. The solution was extracted using ethyl acetate before being washed with deionized water and brine. Subsequent concentration using a rotovap gave a white solid which gives the amine version of the chloroalkane tag. To synthesize the carboxylic acid version of the

chloroalkane tag, the amine version was reacted in 50 mL of dry DCM, N, N-diisopropylethylamine, and 4-dimethyaminopyridine under strong stirring and dry conditions. Succinic anhydride (8.0 mmol) was added to the reaction and allowed to react for two hours at room temperature. Quenching of the reaction was done using 1 M HCl followed by DCM extraction and subsequent wash using deionized water and brine. The organic layers were collected, concentrated via rotovap, and purified using RP-HPLC to give the final carboxylic acid version of the chloroalkane tag (Scheme 5).



Scheme 4. Synthesis of the amine version of the chloroalkane tag is depicted by the first three steps. The fourth step details the synthesis of the carboxylic acid version of the chloroalkane tag.

Deprotection of O-Allyl Glutamic Acid Containing Peptides

One equivalent or the entirety of a peptide was reacted with 0.88 equivalents Tetrakis(triphenylphosphine)palladium(0) and 40 equivalents of phenylsilane in dry DCM under dark conditions for an hour. The product was soaked in 0.02 M sodium dimethyl dithiocarbamate dihydrate in DMF twice for ten minutes each.

Coupling of Chloroalkane Tag and Peptide

One equivalent of deprotected peptide or the entirety of the peptide was reacted with 10 equivalents of both DIC and HOAt, three equivalents of DIEA, and three equivalents of chloroalkane tag. The chloroalkane tag used depends on the placement of the O-Allyl group on the Glu residue.

Chloroalkane Penetration Assay

HeLA cells expressing a HaloTag-GFP-Mito construct were cultured in high glucose DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 μg mL^{-1} puromycin. Cells were seeded into 96-well plates and incubated for 16 hours. The cells were given fresh media as a 1% DMSO stock solution of a chloroalkane-tagged peptide was prepared in fresh media. The chloroalkane-tagged peptides were added to the cells and incubated for four hours at 37°C. Upon completion, the media was aspirated, and the cells were washed with fresh media. Cells were treated with 5 μM chloroalkane tagged TAMRA (tetramethylrhodamine) for 15 minutes. After a wash with fresh media, the cell was trypsinized and resuspended in phosphate-buffered saline. The cells were then analyzed by flow cytometry while gating for live, HaloTag expressing (by measure of GFP fluorescence), and measuring 3000-5000 cells per well. TAMRA fluorescence per well was normalized to untreated cells (0% fluorescence) and cells treated only with chloroalkane-tagged TAMRA (100% fluorescence). All peptides were tested in, at minimum, triplicate (Figure 1).

Chapter 3: Results and Discussion

Six different linear peptides along with their cyclic amide versions were synthesized (Supplemental Table 1; Supplemental Figure 1). The twelve peptides were sent for analysis through chloroalkane penetration assay (CAPA). Different concentrations of the chloroalkane tagged peptides were added into cells and their fluorescence was measured to give CP_{50} values (Figure 4C and 4D). CP_{50} values of the peptides showed linear peptides were more permeable when compared to their cyclic counterparts. This can be seen as the CP_{50} values of the linear peptides are considerably lower than those of the cyclic peptides (Figure 4A and 4B). The only exception to this trend is P2 where the CP_{50} value for the cyclic peptide is lower than its linear counterpart, although the difference was not much. P2 is the only peptide example that followed the well characterized trend of macrocyclic peptides being more permeable than linear peptides. This pointed to the presence of underlying issues as the expectation was for the cyclic peptides to be more permeable than the linear peptides. One potential issue is attributed to the stability of the linear peptides since they are prone to degradation. Premature degradation of the peptide examples could be the reason the CP_{50} values are low. The solution to the issue involves using D-amino acids instead of L-amino acids since the D isomer of amino acids has shown to be more stable. Another issue involves the presence of C-terminal aldehydes which could be increasing their ability to permeate the cell. The solution to this would be to determine the effect of aldehydes on cell permeability by comparing peptides containing them with their amide peptides counterparts. It could be because the cyclic peptides are not as permeable as we thought.

Due to the results of the P2 peptide, it was used to create a second set of peptide examples which included six different peptides: a C-terminal aldehyde peptide made from D-amino acids (P2D-Linear), the same peptide made from L-amino acids (P2), an amide peptide made from D-amino acids (P2D-am), its cyclic version (P2D-AmCyclic), and its CyClick version (P2D).

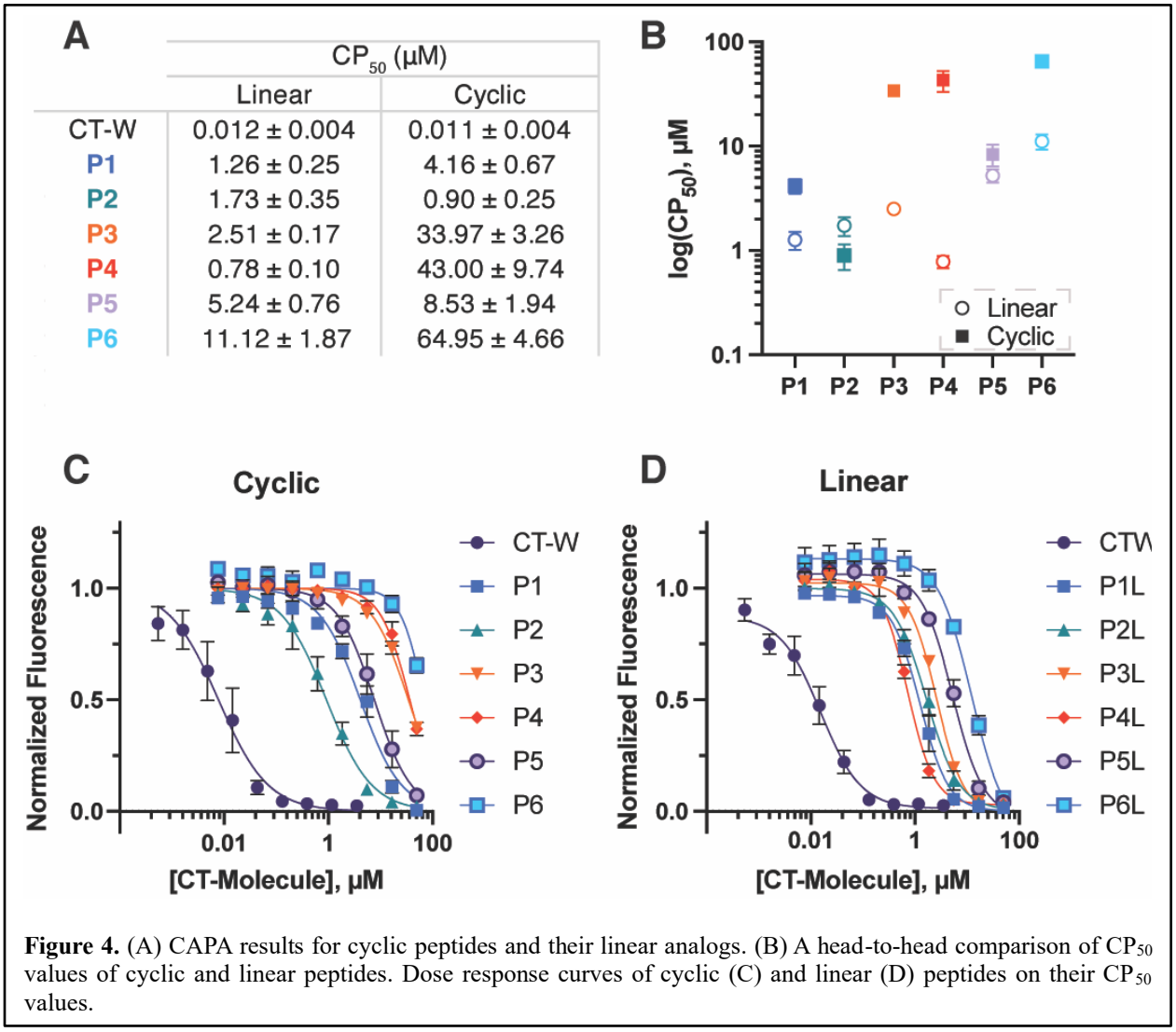


Figure 4. (A) CAPA results for cyclic peptides and their linear analogs. (B) A head-to-head comparison of CP_{50} values of cyclic and linear peptides. Dose response curves of cyclic (C) and linear (D) peptides on their CP_{50} values.

Regarding the presence of aldehydes at the C-terminus of the peptide, those containing the functional group showed lower a CP_{50} compared to their amide linear counterparts. The observation suggests the presence of aldehydes at the C-terminus of peptides increases their cellular penetration. This is possible through an anchoring mechanism to the cell membrane similar

to the previously reported aldehyde anchoring hypothesis.[12] We also wanted to examine the effect of the 4-imidazolidinone moiety on CyClick peptides and their impact on cellular permeability. When comparing P2D-Cyclic and P2D-AmCyclic, the CyClick macrocyclized peptide had a higher CP_{50} than its cyclic amide version (Figure 5A and 5C). In looking at the structures of the macrocyclic peptides, there is a different in their structures as P2D-Cyclic contains a free aspartate side chain thus giving the peptide a -1 charge. P2D-AmCyclic does not have this issue as the aspartate side chain is the site of conjugation to the chloroalkane tag resulting in a neutral molecule. Presence of any charge on a molecule is known to decrease permeability across the cell membrane thus resulting in the higher CP_{50}. Although the difference between them is low signifying that there is a potential for the 4-imidazolidinone functional group to impact permeability.

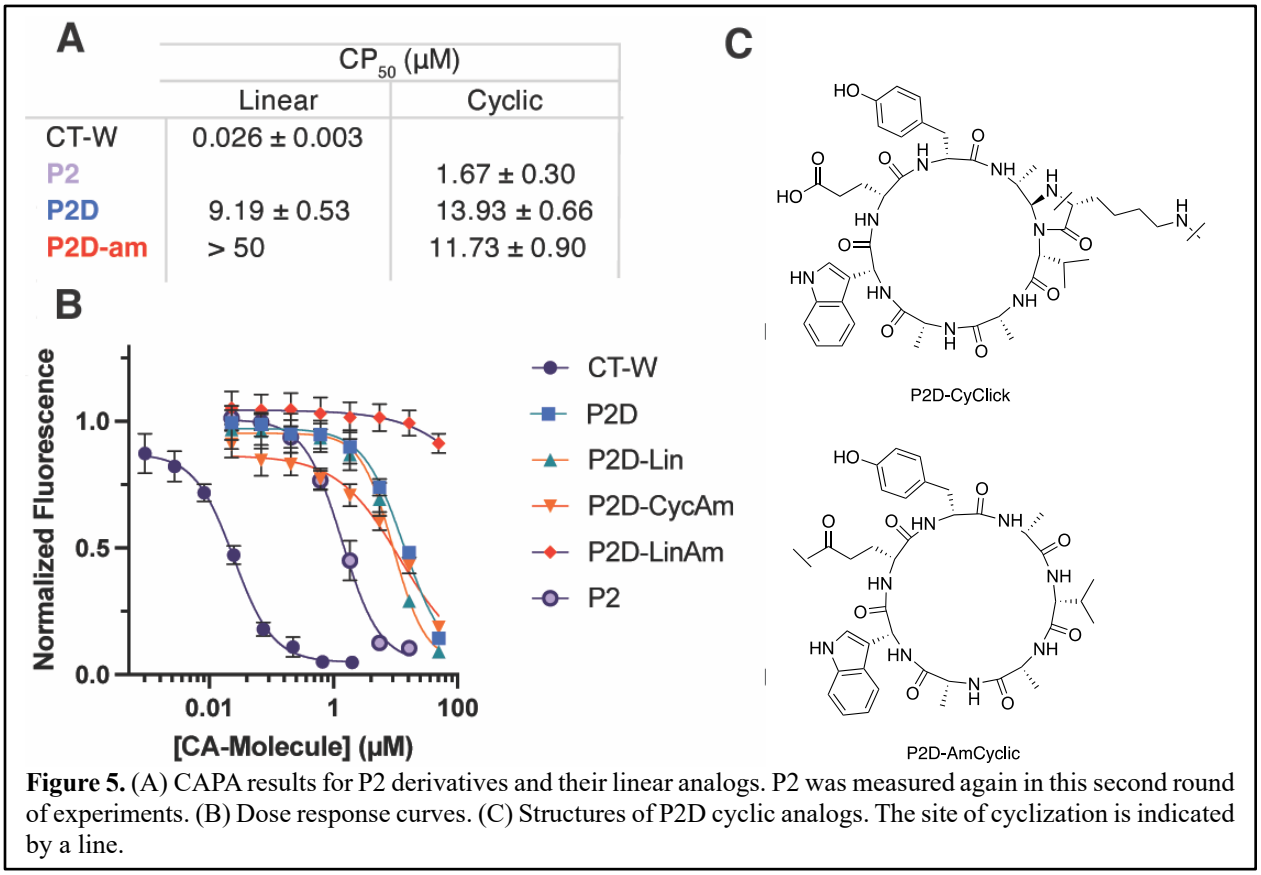


Figure 5. (A) CAPA results for P2 derivatives and their linear analogs. P2 was measured again in this second round of experiments. (B) Dose response curves. (C) Structures of P2D cyclic analogs. The site of cyclization is indicated by a line.

A third set of peptides examples were synthesized to confirm the results discovered through the second set using P2. We wanted to confirm if the presence of an aldehyde on the C-terminus

of the aldehyde universally increases the cellular permeability of linear peptides. Using the C-terminal aldehyde version of the linear peptides from the first set, CAPA was performed (Figure 3). CAPA results demonstrate lower CP_{50} for peptide aldehydes when compared to peptide amides (Am), peptides with a C-terminus (COOH) (Figure 6). The trend holds for three out of the four peptides examined, the lone exception being P4D. P4D did have a lower CP_{50} for the peptide aldehyde, but the results were not significant. These results suggest that the presence of aldehydes on the C-terminus of peptides results in overall increased cell permeability.

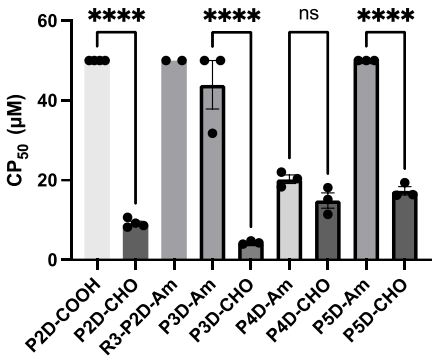


Figure 6. CAPA results for C-terminal amide and aldehyde peptides comparing CP_{50} values.

We also wanted to confirm if the 4-imidazolidinone moiety impacts cellular permeability as previously suggested. This was done using two 18-atom macrocyclic peptides with identical side chain composition, the difference being cyclization through CyClick chemistry (Figure 7A). CAPA was performed on the two peptides showing the CyClick peptides having a measured CP_{50} of 8.03 μM and the amide macrocycle being 15.7 μM (Figure 7B and 7C).

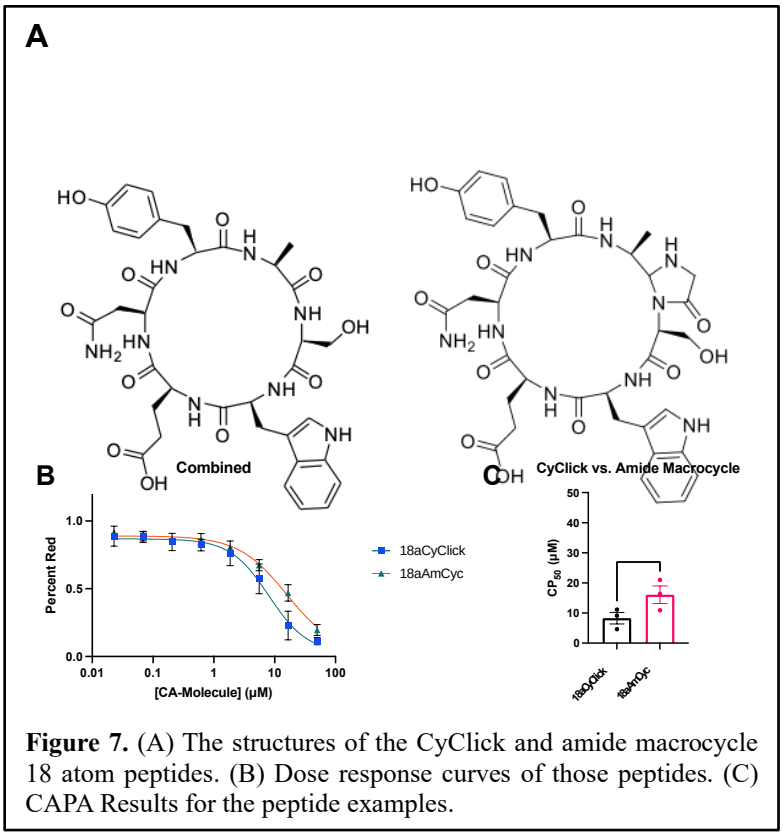


Figure 7. (A) The structures of the CyClick and amide macrocycle 18 atom peptides. (B) Dose response curves of those peptides. (C) CAPA Results for the peptide examples.

The CyClick peptide is almost two times as permeable compared with the amine macrocycle, demonstrating that the 4-imidazolidinone functional group increases the cellular permeability of peptides.

After looking at peptide aldehydes and macrocyclic peptides, we wanted to examine three questions relating to peptide aldehydes. How does the presence of aldehydes on cyclic peptides affect their permeability? How does the presence of multiple aldehydes impact the permeability of peptides? How does the combination of both the aldehyde and the 4-imidazolidinone moiety impact the permeability of a peptide? A series of six macrocyclic peptides were made: two controls (P7-Gln and P8-Gln), one containing an aldehyde group (P7-CHO), one containing two aldehyde groups (P8-CHO), one containing an aldehyde and a glutamate residue (P8-Mix), and one containing both an aldehyde and 4-imidazolidinone (P8-CyClick-CHO; Figure 8).

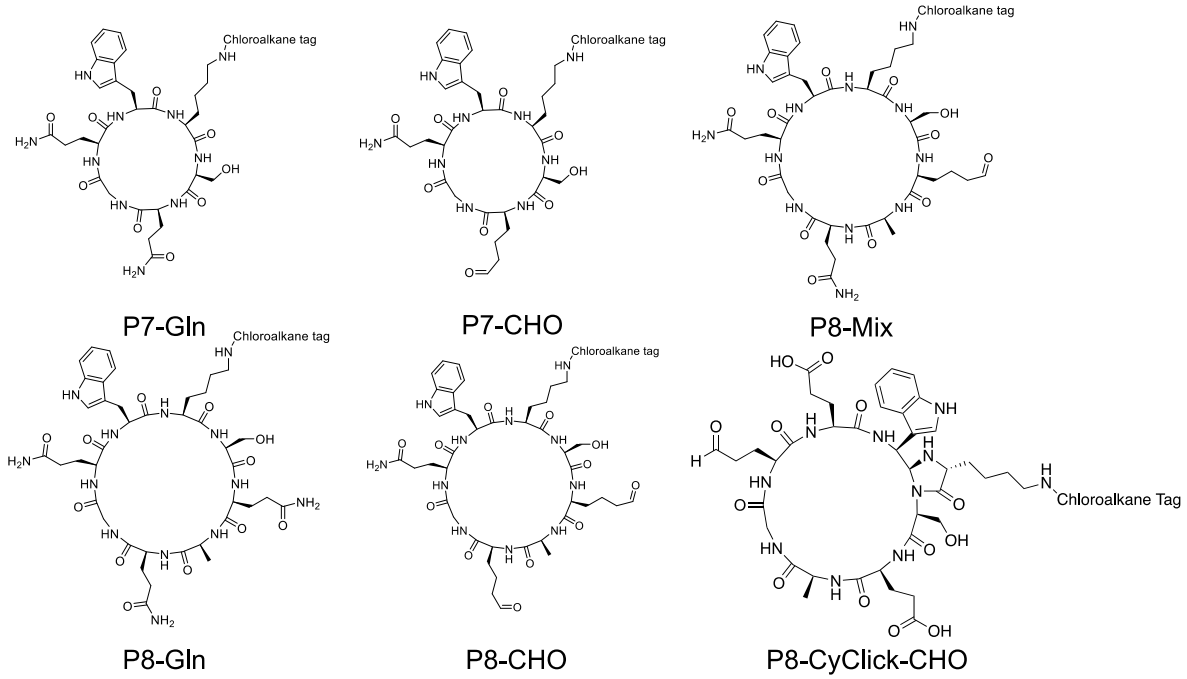


Figure 8. The structures of the fourth set of peptide examples focused on macrocyclic peptide aldehydes.

The synthesis of these peptides was different due to the presence of the aldehyde on a sidechain instead of a terminal end. We incorporated a dimethyl lysine residue during SPPS as the Raj group

has developed a reaction that selectively converts dimethyl lysine to an aldehyde (Figure 9). The method involved reacting the peptide with pyridine and 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate) (Selectfluor™).

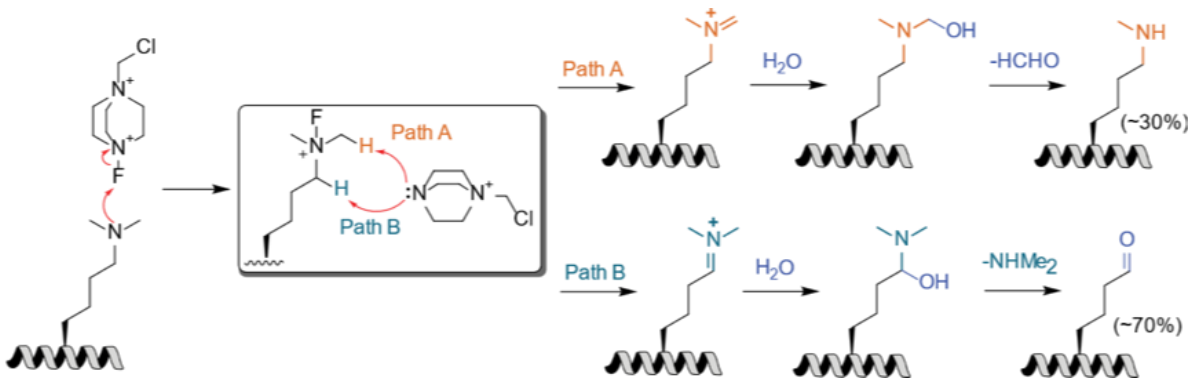
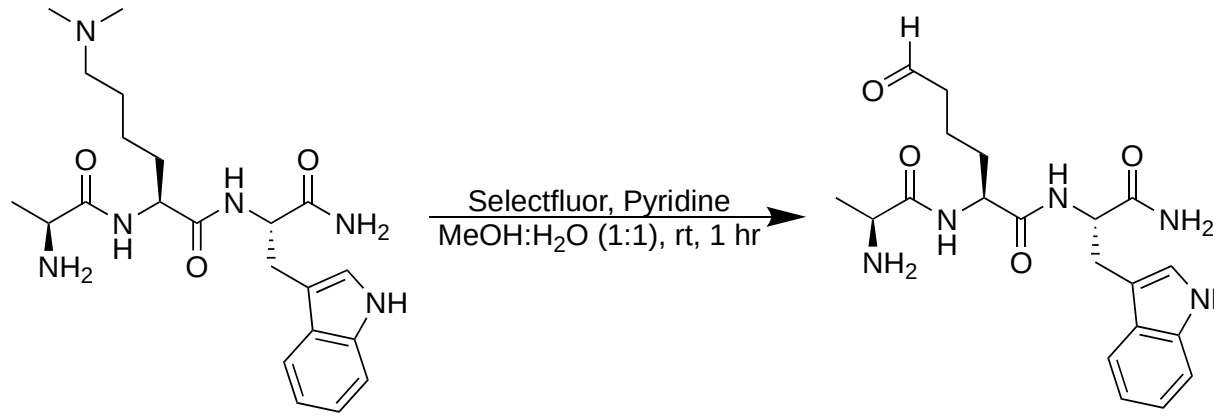


Figure 9. Selectfluor™ reacts with a dimethyl lysine residue to go on one of two pathways depending on where an imine is formed. Subsequent reaction with water results in either the formation of the aldehyde or a demethylated lysine.

This reaction had only previously been done in solution where a tryptophan residue is oxidized and fluorinated in addition to the formation of the aldehyde. We wanted to avoid this issue and optimize this reaction by doing it on-resin where all the other amino acid residues are protected. In optimizing this reaction, we used a model peptide (MW: 429.26) that would undergo various reaction conditions (Supplemental Scheme 1). Five conditions were examined: (1) 4 equivalents Selectfluor™ and 20 equivalents pyridine in 80% DMF/20% water, (2) 4 equivalents Selectfluor™ and 20 equivalents pyridine in 80% THF/20% water, (3) 4 equivalents Selectfluor™ and 20 equivalents pyridine in 80% ACN/20% water, (4) 3 equivalents Selectfluor™ and 15 equivalents pyridine in 90% DMF/10% water, (5) 3 equivalents Selectfluor™ and 15 equivalents DMAP in 90% DMF/10% water. Using mass spectrometry, we examined to see if the different reaction conditions and saw a mix of results. For condition A, we were able to observe the starting

material at 431 m/z and resin fragments at 261 m/z. Other masses are in low abundance and do not correspond to either the starting material, demethylated lysine, or the aldehyde product (Supplemental Figure 2A). For condition B, we also see the presence of a resin fragment and a tiny peak corresponding to our product (Supplemental Figure 2B). For condition C, there appeared to be no reaction as the starting material is in high abundance (Supplemental Figure 2C). For condition D, we once again saw a resin fragment of the product and a small peak at 401 m/z corresponding to our aldehyde product (Supplemental Figure 2D). For condition E, there was a mix of products that did not correspond to anything other than potential resin fragments and our starting material (Supplemental Information 2E). Based on these initial results, we wanted to do further analysis on conditions B and D by running these reactions on an HPLC (Supplemental Figure 3A and 4A). For condition B, we collected fractions from the HPLC at three different time points to determine where our peptide aldehyde would be found. When looking at the mass spectrometry data, the demethylate lysine was present at 417 m/z in addition to resin fragments and a little bit for the starting material (Supplemental Figure 3B, 3C, and 3D). For condition D, there was the presence of the starting material, various resin fragments of the various peptides, and the desired peptide aldehyde (Supplemental Figure 4B, 4C, 4D, and 4E). From these conditions, the peptide aldehyde was observed but it was done so in low abundance and along with other products. We could not achieve a complete and clean conversion from our starting material to the aldehyde product. As a result, we decided to do the aldehyde formation reaction in solution with the intent of making our peptides with both the aldehyde on the side chain and the modified tryptophan residue (Scheme 5). The peptides have been made and are in the process of CAPA analysis by the Kritzer group.



Scheme 5. Dimethyl lysine is converted to a peptide aldehyde in solution.

Chapter 4: Conclusions and Future Directions

Through the analysis of the first set of peptides, the lower CP_{50} that the linear peptides had were attributed to two factors: the stability of the peptides and the presence of aldehydes at the C-terminus of the linear peptides. To synthesize the first set of peptides, L-amino acids were used. This made them susceptible to proteolytic degradation in cells, resulting in the absorbance of chloroalkane tag only leading to the lower CP_{50}. As for the issue involving peptide aldehydes, a second set of peptides was synthesized using one of the peptides from the first set, P2. Various analogs of P2 were synthesized to look at the effect of aldehydes and a 4-imidazolidinone moiety on the cell permeability of peptides. The data from CAPA analysis of those peptides suggests that aldehydes increase permeability. The 4-imidazolidinone group was found to have the potential to impact permeability due to its ambiguity regarding its CP_{50} values and the charge of the CyClick peptide. Another set of peptides was examined to give proof of the use of aldehydes and CyClick-synthesized peptides on cell permeability. CAPA analysis demonstrated that aldehydes increase cellular permeability in linear peptides. It also depicted CyClick peptides containing the 4-imidazolidinone moiety to have higher permeability than its amide macrocycle counterpart.

To further study the impact of these functional groups on peptide permeability in cells, the last set of peptide examples including the macrocyclic peptide aldehydes need to be examined through CAPA. This will indicate if aldehydes can universally increase the permeability of peptides, whether linear or cyclic. Additionally, these functional groups need to be compared to other groups used to increase cell permeability such as a polyarginine and a TAT sequence.

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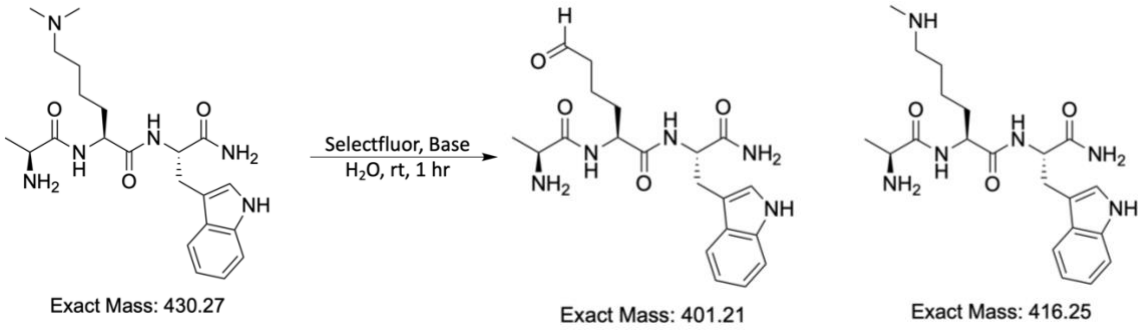
Supplemental Information

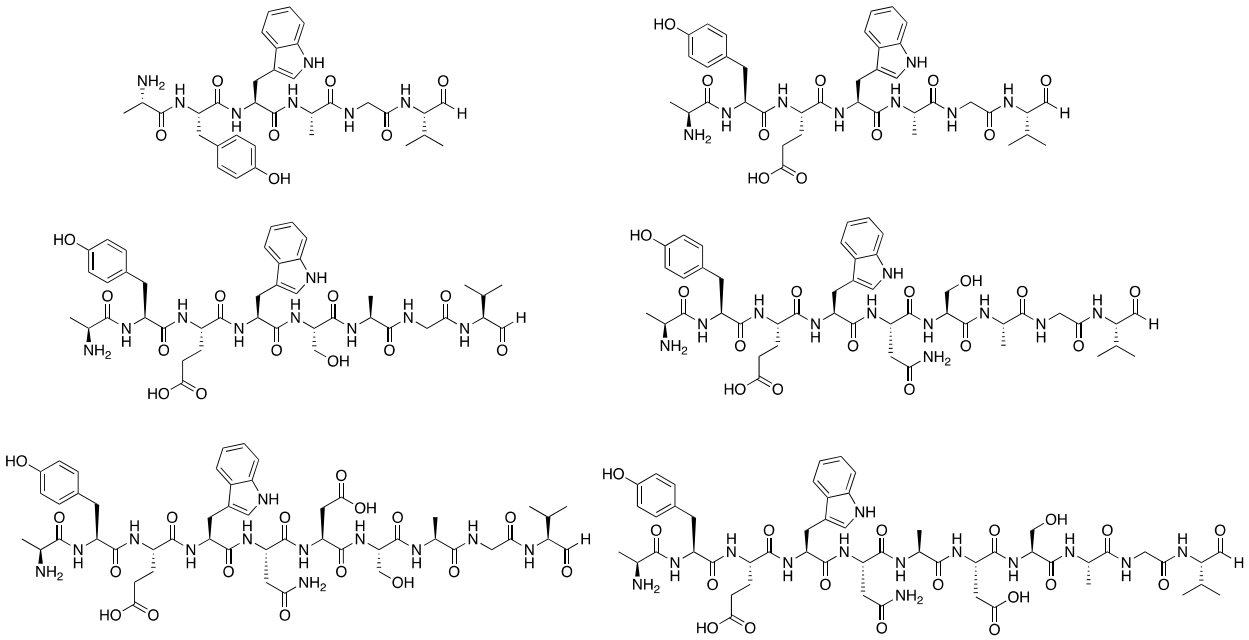
Supplemental Table 1. Peptides used throughout the studies. All peptides were made containing the chloroalkane tag. These are also the same sequences for their cyclic counterparts. All KMe_2 residues corresponds to aldehydes.

Name	Sequence
P1	N-VGAWYA-C
P2	N-VGAWEYA-C
P3	N-VGASWEYA-C
P4	N-VGASNWEYA-C
P5	N-VGASNDWEYA-C
P6	N-VGASNADWEYA-C
P2-CHO	N-VGAWEYA-CHO
P3-CHO	N-VGASWEYA-CHO
P4-CHO	N-VGASNWEYA-CHO
P5-CHO	N-VGASNDWEYA-CHO
18mer Cyclic Peptide	N-SWENYA-C
P7-Gln Cyclic	N-GQSKWQ-C
P8-Gln-Cyclic	N-GQAQSKWQ-C
P7-CHO-Cyclic	N-G(KMe_2)SKWQ-C
P8-Mix-Cyclic	N- GQA(KMe_2)SKWQ-C
P8-CHO-Cyclic	N- G(KMe_2)A(KMe_2)SKWQ-C

Supplemental Scheme 1. Conversion of the model peptide containing dimethyl lysine to either the peptide aldehyde or the demethylated lysine. Structures include the m/z that corresponds to their expected m/z values using mass spectrometry.

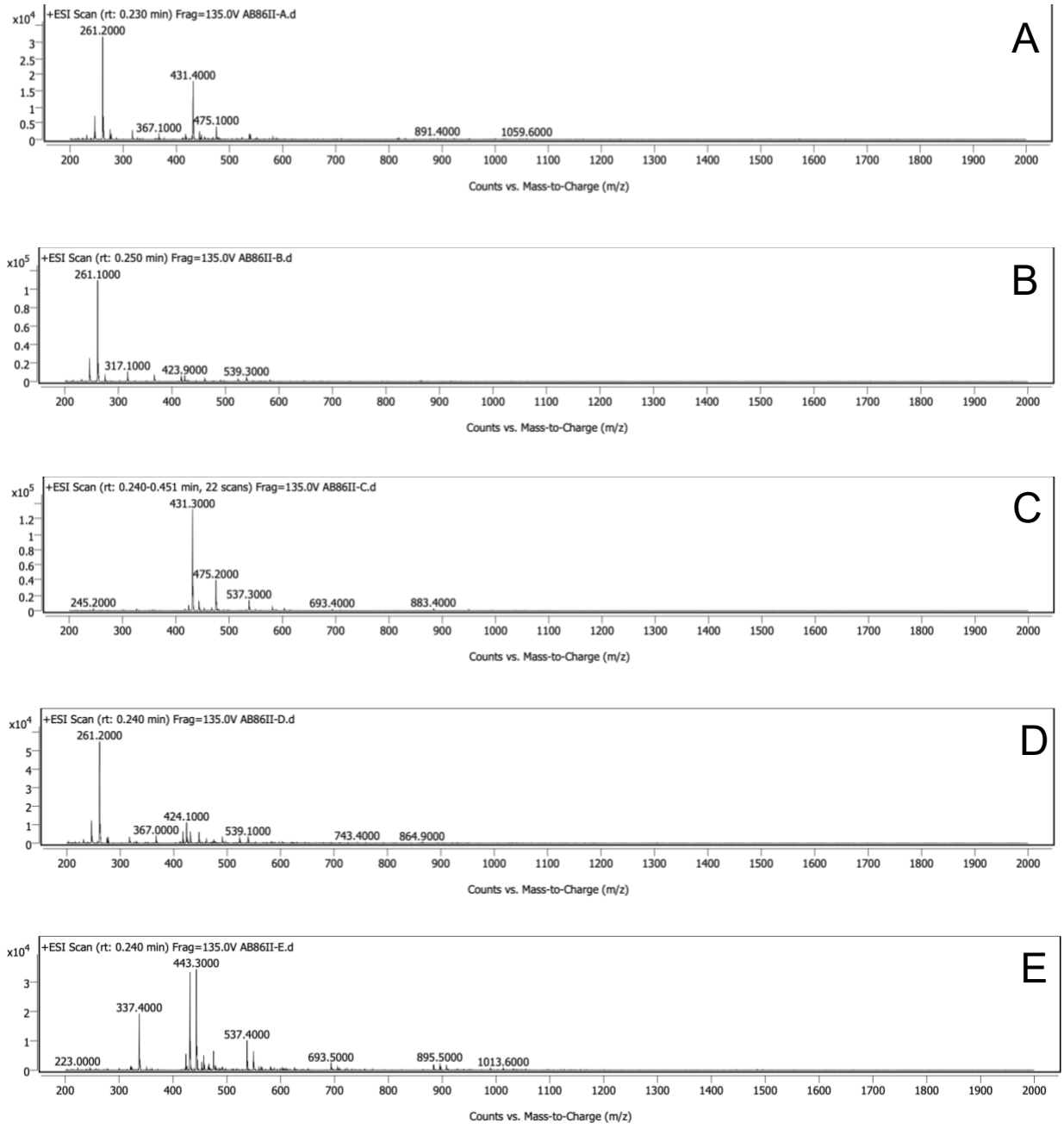


Supplemental Figure 1. The structures of the P1 (top left), P2 (top right), P3 (middle left), P4 (middle right), P5 (bottom left), P6 (bottom right) used in the first set of CAPA analysis.

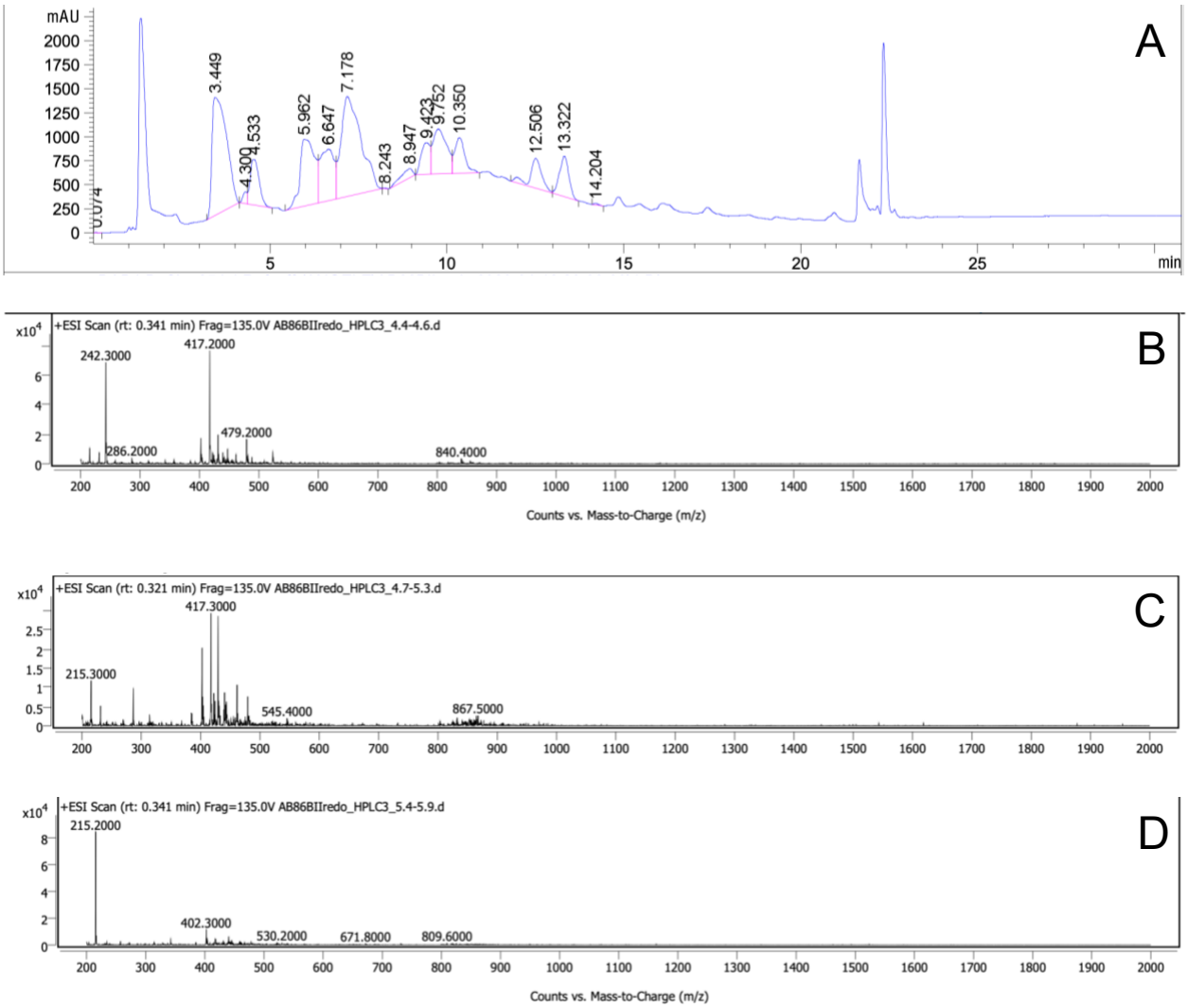


Supplemental Figure 2. The mass spectrometry data from the five reaction conditions tested to see conversion of our model peptide into the desired peptide aldehyde. (A) 4 equivalents

Selectfluor™ and 20 equivalents pyridine in 80% DMF/20% water, (B) 4 equivalents Selectfluor™ and 20 equivalents pyridine in 80% THF/20% water, (C) 4 equivalents Selectfluor™ and 20 equivalents pyridine in 80% ACN/20% water, (D) 3 equivalents Selectfluor™ and 15 equivalents pyridine in 90% DMF/10% water, (E) 3 equivalents Selectfluor™ and 15 equivalents DMAP in 90% DMF/10% water.



Supplemental Figure 3. Follow-up on condition B. (A) HPLC trace for condition B. The mass spectrometry results for the fractions collected at times (B) 4.4-4.6 minutes (C) 4.7 – 5.3 minutes, and (D) 5.4 – 5.9 minutes.



Supplemental Figure 4. Follow-up on condition D. (A) HPLC trace for condition B. The mass spectrometry results for the fractions collected at times (B) 4.4-4.9 minutes (C) 4.9 – 5.4 minutes, (D) 6.1 – 6.8 minutes, and (E) 6.8-7.3