SYNTHESIS OF A CATIONIC AMPHIPHILIC POLYPROLINE HELIX (CAPH) CONJUGATE WITH POLYMYXIN B

by

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Dedicated to my family and best friends, who have been my rock throughout this journey

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ABSTRACT

Pathogens such as *Listeria, Shigella*, *Brucella*, *Salmonella*, *Mycobacterium tuberculosis* and *methicillin-resistant Staphylococcus aureus* (MRSA) can traverse into mammalian cells, such as phagocytic macrophages. Once inside, these bacteria can survive and reproduce, causing chronic infections. It is of utmost importance to develop novel antibiotics with broad spectrum activity to control these deadly bacteria. Broad spectrum activity will allow for targeting of pathogens with different structures and cell membrane components.

This work focuses on the synthesis of a dual antibiotic agent, composed of a cationic amphiphilic polyproline helix (CAPH) possessing cell penetrating and nonmembrane lytic antimicrobial capabilities (P14LRR), and a derivative of the polymyxin B (PMX) antibacterial peptide. This dual antibiotic conjugate was created to be a tool to potentially clear intracellular pathogenic bacteria. Overall, the reduction of the disulfide bond linking the two antibiotics within the reducing environment of cells would release the individual antimicrobial agents, and could have improved cell membrane penetration and intracellular synergistic activity. Herein, the synthesis of the dual antibiotic agent, **P14LRR-PMX**, is discussed.

CHAPTER 1. SYNTHESIS OF A P14LRR-CYS-POLYMYXIN PEPTIDE ANTIBIOTIC CONJUGATE

1.1 Introduction

Bacterial infections have been a consistent threat to human life for as long as they have existed. The discovery of antibiotics provided protection against many pathogens, with hope for life after an infection. However, drug resistance allowed these dangerous pathogens to produce mechanisms to inhibit the activity of previously functional antibacterial agents. These mechanisms include the use of active efflux pump systems, limitations on drug uptake, modifications to the drug target, and drug inactivation; all of which can result in the development of antibiotic resistance.[1] Every year, a minimum of 2.8 million people contract an antibiotic-resistant infection in the United States alone; of those 2.8 million, more than 35,000 lose their life. Pathogens such as *Listeria, Shigella, Brucella, Salmonella, Mycobacterium tuberculosis* and *methicillin-resistant Staphylococcus aureus* (MRSA) reside within mammalian cells like phagocytic macrophages and reproduce, causing chronic infections.[2] Drug selectivity, accumulation and cell penetration often serve as major roadblocks in the treatment of these illnesses.

Many classes of antibiotics are available to control and eradicate these pathogens, such as β-lactams which inhibit cell wall synthesis, lipopeptides which depolarize cell membranes and tetracyclines which inhibit protein synthesis.[1] However, bacteria have been able to develop resistance to many of these existing therapeutics. Currently, very few FDA approved antibiotics of last resort remain, including carbapenem and colistin, as well as the widely known vancomycin.[3] To provide broader coverage against Gram-positive and Gram-negative pathogens, novel agents need to be introduced to the market that display broad spectrum activity and can target multi-drug resistant pathogens.

Among the therapeutics being explored for this purpose are antimicrobial peptides (AMPs). They are a class of antibiotics which have been shown to treat bacterial infections, however, a disadvantage of using these therapeutics is their lytic mechanism of action. In this mode of action, the cell membrane of pathogens is targeted, leading to microbial cell lysis while also resulting in the cytotoxicity of the host cells.[4] Recently, the Chmielewski group has explored proline-rich AMP-inspired analogues which are non-membrane lytic and can localize inside mammalian cells.[5,6,7,8,9] These peptides possess a polyproline type II helical (PPII) conformation and were

designed to contain specific hydrophobic and cationic residues.[10] The first peptide has a sequence with four copies of the PRP triad repeat which can be found in PR-39 – this was Fl-PRP-4 (fluorescein was placed at the N-terminus following a glycine spacer).[1,10a] A more cationic PRR triad was also applied in order to improve mammalian cell uptake, producing Fl-PRR-4. This peptide was then modified to comprise all proline-based residues along with a guanidium group-having unnatural amino acid (P_R) in order to probe the relationship between activity and the ability to form the PPII conformation.[5] The resulting peptide, Fl-PP_RP_R-4, was altered further to include an isobutyl-modified proline unnatural amino acid (P_L) which would add hydrophobicity to this design. This was done to produce two peptides with different lengths, Fl-$P_LP_RP_R$-3 and Fl-$P_LP_RP_R$-4 (Figure1).[5]

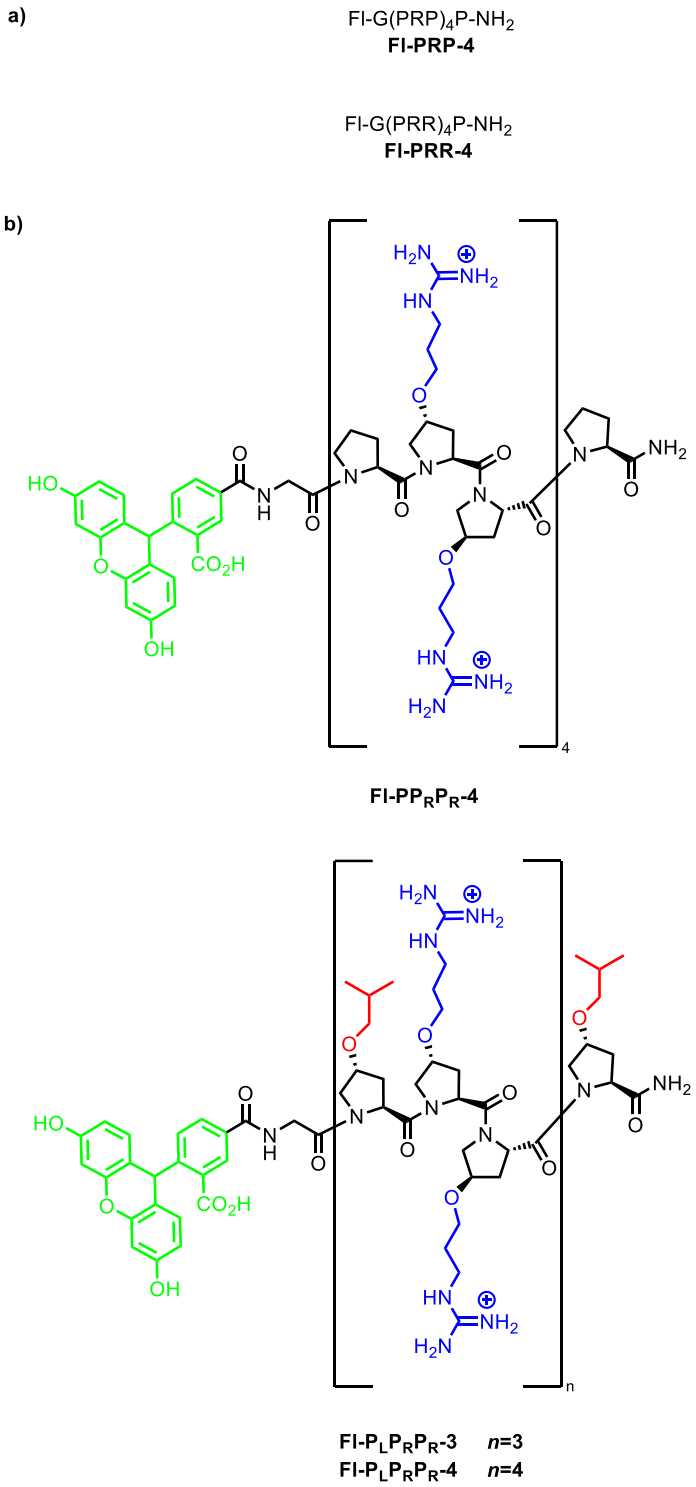


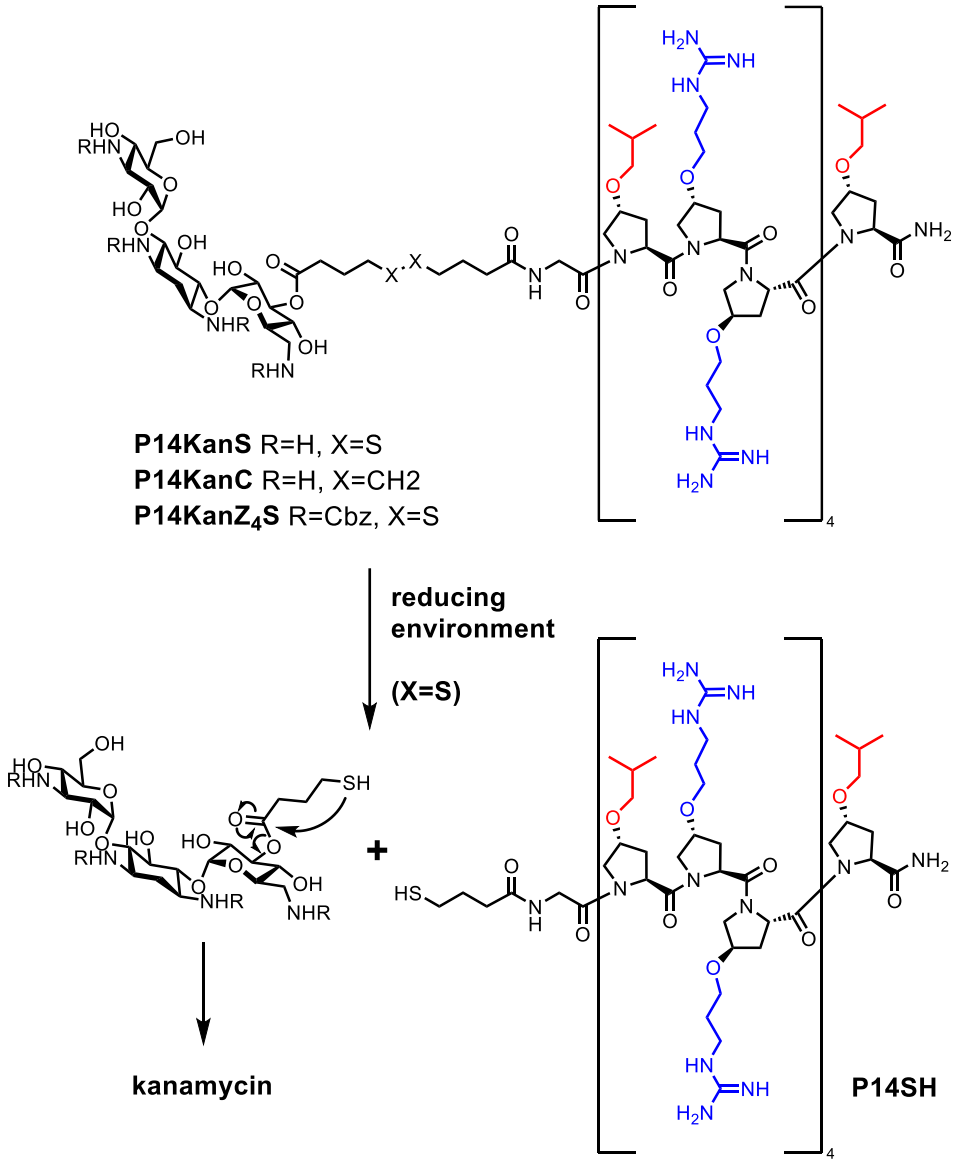
Figure 1: Structures of the five P-AMPs investigated; (a) peptides containing natural amino acids, (b) peptides containing unnatural proline derivatives, P_R (in blue) and P_L (in red).[5]

Once the five peptides were in hand (Figure 1), their antibacterial activity against *E. coli* and *S. aureus* was explored. Fl-PRP-4 was found to be inactive against both strains while Fl-PRR-4, which is slightly more cationic, showed some observable activity in Gram-positive and Gram-negative strains. Similarly, going from Fl-$P_LP_RP_R$-3 to Fl-$P_LP_RP_R$-4, with the increase in cationic character an increase in antibacterial activity was also observed. Fl-$P_LP_RP_R$-3 displayed MICs of 60 μM for both *E. coli* and *S. aureus*.[5] In contrast, Fl-$P_LP_RP_R$-4 had MICs of 4 μM and 12 μM, respectively. This supports the idea that electrostatic interactions between cationic antimicrobial peptides and the negatively charged bacterial membranes of the pathogens they target are essential for antibacterial activity. Comparing Fl-PP_RP_R-4 with Fl-$P_LP_RP_R$-4, it was found that the introduction of hydrophobic isobutyl groups improved the activity for both bacteria.[5] The proline-rich peptides with de novo scaffolds displayed minimal damage to hRBC human red blood cells and Fl-$P_LP_RP_R$-4 proved to be the non-hemolytic antibacterial agent with the highest potency among all five peptides studied.[5] The cell penetrating abilities of the most cationic peptides in J774A.1 macrophage cells were studied and a four-fold increase in uptake going from Fl-PRR-4 to $P_LP_RP_R$-4 was observed.[5] Additionally the latter was internalized the best within these cells, while also displaying low toxicity and showing resistance to proteolytic degradation with trypsin.[5]

The subcellular localization of the five peptides was studied by confocal microscopy. Fl-$P_LP_RP_R$-4 was present in both endosomes and the mitochondria of J774A.1 cells.[5] Fl-$P_LP_RP_R$-4 showed the best antibacterial activity and macrophage penetration, and was therefore selected for testing against various bacterial pathogens.[5] This peptide was effectively able to inhibit the growth of *Listeria, Salmonella,* and *Brucella* at varying concentrations, while eradicating the latter two in J774A.1 cells by 62% and 90%, respectively.[5] The effect of this peptide on membrane integrity was also investigated with a β-galactosidase leakage assay and no leakage was observed denoting bacterial membrane lysis is not part of the peptides mechanism of antibacterial action.[11,12]

The inability of many regularly used antibiotics to accumulate within macrophage cells can inhibit their antibacterial activity.[13] Some factors that affect this accumulation include unsuccessful penetration into the membrane and exposure to drug efflux transporters which eject the drug from the membrane.[13] Liposomes and micro/nanoparticles have been explored as effective delivery vehicles for antibiotics in targeting intracellular bacteria.[14] However, their instability in biological fluids and drug loading challenges limits their use.[15,16] The previously discussed nonmembrane lytic, cell-penetrating peptide with broad spectrum activity, Fl-$P_LP_RP_R$-4 (P14LRR),

was investigated by the Chmielewski group as a dual antimicrobial with the aminoglycoside antibiotic kanamycin, with the aim of achieving a conjugate with synergistic activity (Scheme 1).[8] P14LRR and kanamycin were linked using a tether containing a disulfide bond and one where a carbon-carbon bond was employed instead, forming P14KanS and P14KanC, respectively.[8] In the reducing environment within mammalian cells, P14KanS was expected to release kanamycin and P14SH after reduction of the disulfide bond.[8] The half-life of release of this conjugate was investigated using P14KanZ_4S (Scheme 1), and this agent released KanZ_4 with a half-life of 1.5±0.2 h. P14KanC however, experiences no changes in a reducing environment.[8]



Scheme 1: Tethered dual antibiotic consisting of the cell penetrating and nonmembrane lytic antibiotic P14LRR, and the antibiotic kanamycin. Within the reducing environment of mammalian cells, P14KanS undergoes disulfide bond reduction, releasing kanamycin and P14SH.[8]

The cellular accumulation of the peptides was investigated using a fluorescent analogue (P14KanC-Fl) in J774A.1 cells and a pattern of concentration-dependent cell penetration was observed.[8] A 20% increase in cell uptake was seen when the fluorescent analogue was compared to P14LRR. The P14LRR peptide was previously found to localize in the endosomes and mitochondria of J774A.1 cells. However, the subcellular localization of the kanamycin conjugate presented it throughout the cell, including the endosomes, cytosol and nucleus.[8] The antimicrobial

activity of P14KanS and P14KanC was probed with various Gram-positive and Gram-negative bacteria. P14KanS proved to be very active across the bacterial types, with a 2- to 16- fold increase in potency when compared to the nonreducible P14KanC and a 2- to 32- fold increase in comparison to the activity displayed by a noncovalent mixture of P14LRR and kanamycin.[8] Compared to P14LRR alone, an 8- to 16- fold increase in antimicrobial activity was observed. Moreover, through a β-galactosidase assay the reducible and nonreducible conjugates presented no disruption to bacterial membranes even at five times their MIC values.[8] These conjugates do not lyse bacterial membranes and show low cytotoxicity in J774A.1 cells at varying concentrations. The cells were also utilized to probe the ability of the conjugates to eradicate intracellular pathogenic bacteria including *S. enteritidis, B. abortus, M. smegmatis, M. tuberculosis,* and *S. flexneri*.[8] A 1:1 mixture of P14LRR and kanamycin displayed decent reduction levels in infected cells containing mycobacteria when compared to each component acting alone. The P14KanS conjugate showed excellent activity against *M. smegmatis* and *M. tuberculosis*, lowering levels of these bacteria by 95% and 93%, respectively. Similarly, this reducible conjugate displayed reductions in *Salmonella, Brucella* and *Shigella* of 95%, 85% and 70%, respectively. In contrast, P14KanC displayed antibacterial activity similar to that of the 1:1 mixture in *Salmonella, Brucella* and *Shigella*, which highlights the importance of the reducible linker in the activity of the conjugate. The combination of the cell penetrating and nonmembrane lytic antimicrobial capabilities of P14LRR with kanamycin creates a tool with significant activity against intracellular pathogenic bacteria.[8] Overall, reduction of the disulfide bond in order to release the two antibiotics results in improved cell membrane penetration, intracellular accumulation and allows the agents to carry out their individual functions synergistically.

Polymyxin B (PMX) is a fast acting lipopeptide bactericidal agent which belongs to the Polymyxin and Colistin family of natural products.[17] Its structure contains a polycationic peptide ring with a tripeptide side chain and a fatty acid tail (Figure 2).[18] Polymyxin B's mechanism of action involves using its polycationic peptide ring to bind to the outer membrane in Gram-negative bacteria, and disrupting the calcium and magnesium bridges that stabilize the lipopolysaccharide (LPS).[18] Since Polymyxin B has an affinity for LPS which is almost 3 orders of magnitude higher than Ca^{2+} or Mg^{2+}, it can displace these ions, disrupting the outer membrane.[18] The physicochemical effect produced by the antibiotic leads to permeability changes, creating openings in the outer membrane of Gram-negative bacteria resulting in cell death.

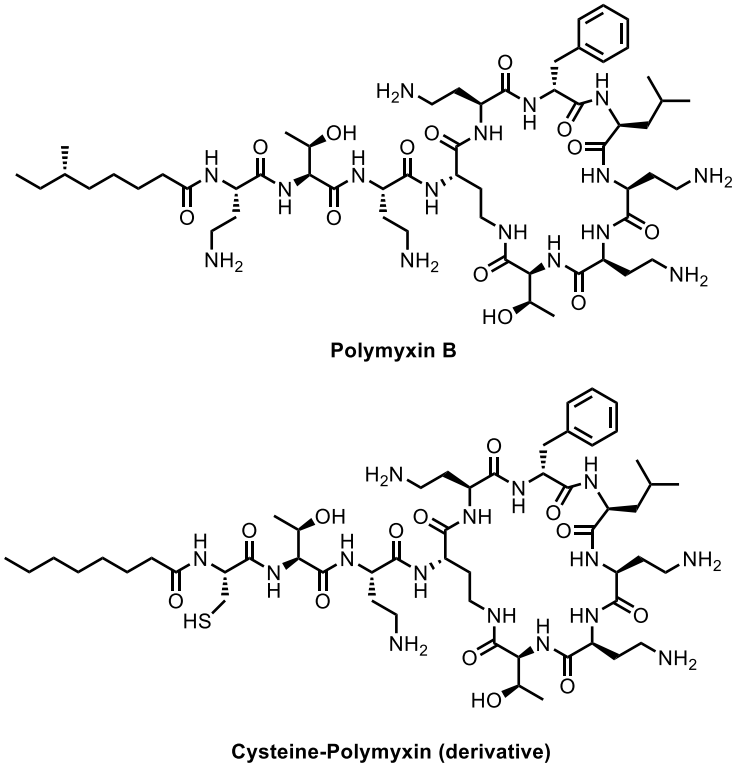


Figure 2: Structure of unmodified polymyxin B (PMX B) and the modified derivative cysteine-polymyxin (PMX-SH).

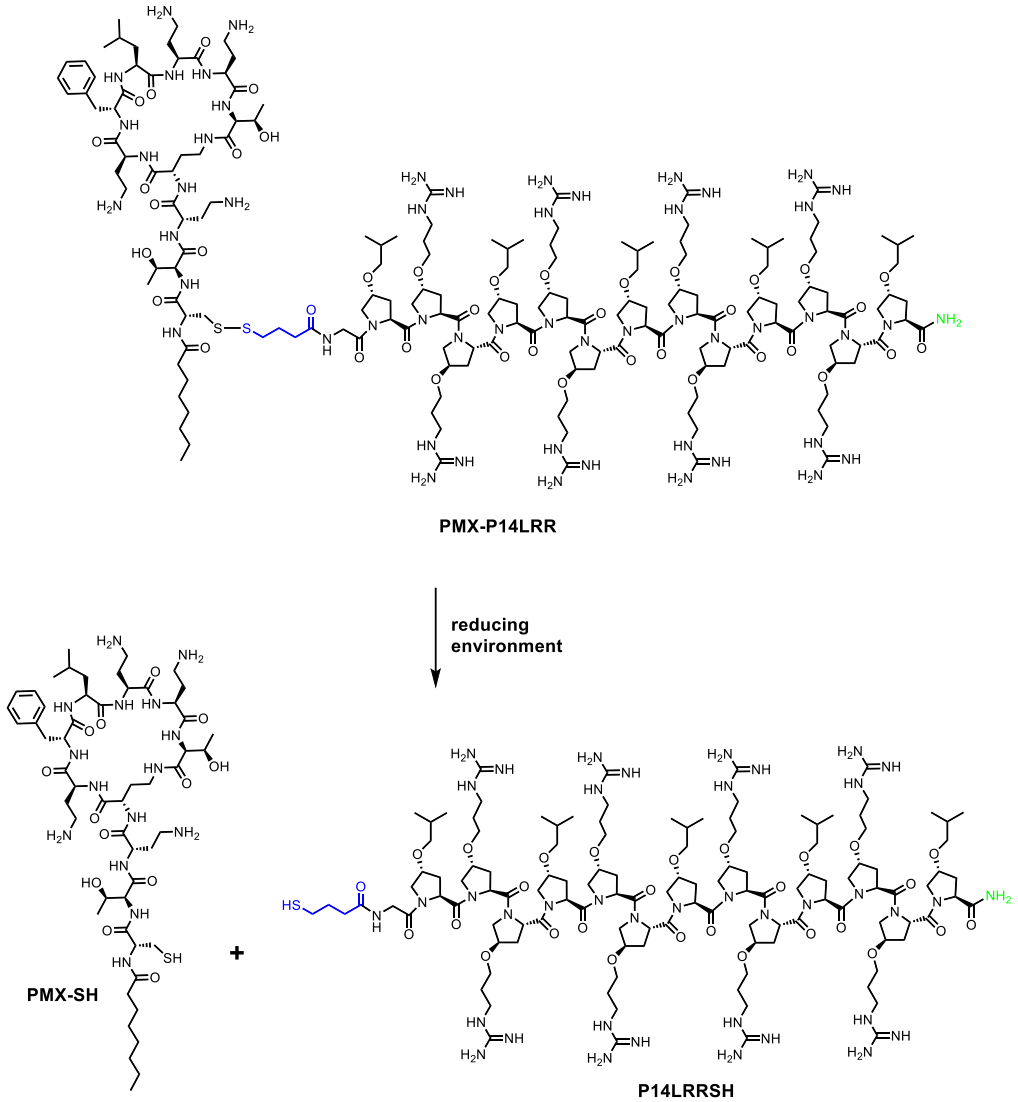
The agent P14KanS presented a dual antibiotic system composed of the cell penetrating and nonmembrane lytic antimicrobial P14LRR and the aminoglycoside antibiotic kanamycin, which within the reducing environment of mammalian cells would release kanamycin and P14SH. These were then able to synergistically carry out individual mechanisms to clear pathogenic bacteria. Similarly, the goal of this work was to create a dual antimicrobial therapeutic composed of P14LRR and PMX which within cells should release P14LRRSH and PMX. In order to execute the conjugation reaction, the α,γ-diaminobutyric acid was exchanged for a cysteine group (Figure 2). This allowed for conjugation of Cys-polymyxin (PMX-SH) to P14LRR through a disulfide bond.

1.2 Results and Discussion

1.2.1 Design

We set out to synthesize the conjugate P14LRR-PMX, which would implement the antimicrobial activity of both agents while utilizing the cell penetrating and nonmembrane lytic

antibiotic properties of the of the CAPH peptide P14LRR. P14LRR would act as a delivery vehicle and can carry PMX to its sites of action. The dual conjugate was then expected to separate into two agents, P14LRRSH and PMX-SH, in the reducing environment of mammalian cells. After this, the antibiotics should perform their separate modes of action at their respective sites within the cells.

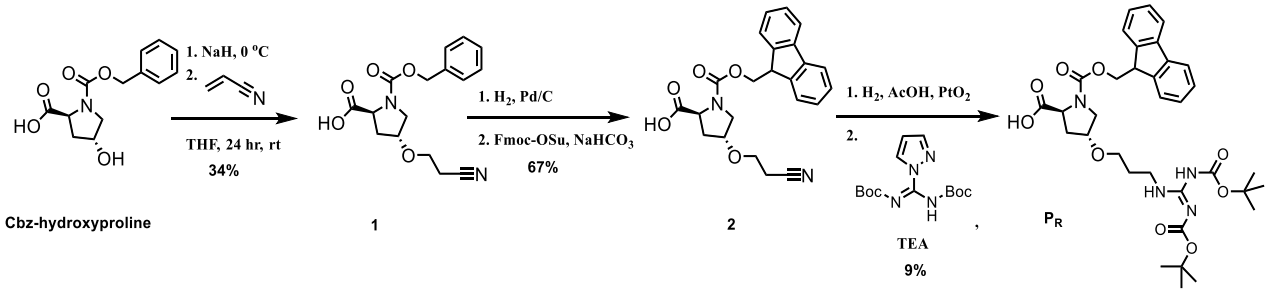


Scheme 2: Release of P14LRRSH and PMX-SH in a reducing environment.

1.2.2 Synthesis

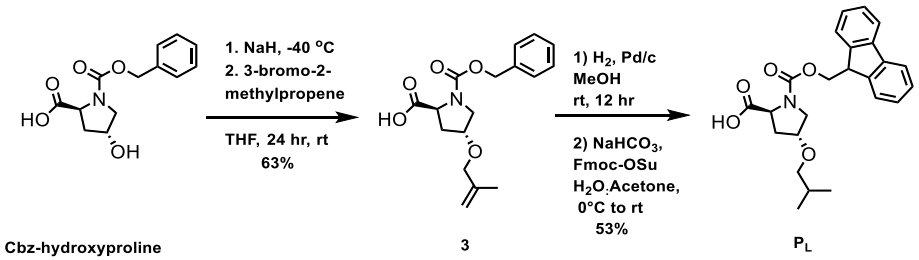
1.2.2.1 Synthesis of P14LRR Peptide

The unnatural amino acid components of P14LRR-Fmoc were synthesized from Cbz-hydroxyproline through two similar pathways as has previously been reported.[6,9] In order to furnish P_R, the cationic amino acid, Cbz-hydroxyproline was treated with NaH and acrylonitrile to provide **1**. This was followed by hydrogenation with palladium on carbon, to generate a 2° amine that was reacted with Fmoc-OSu to provide **2**. Lastly, the nitrile group was reduced, and the resulting 1° amine was reacted with *N,N'*-Di-Boc-1*H*-pyrazole-1-carboxamidine to afford P_R (Scheme 3). The overall percent yield of P_R was low at 9%, compared to the 62% yield reported in literature.[6]



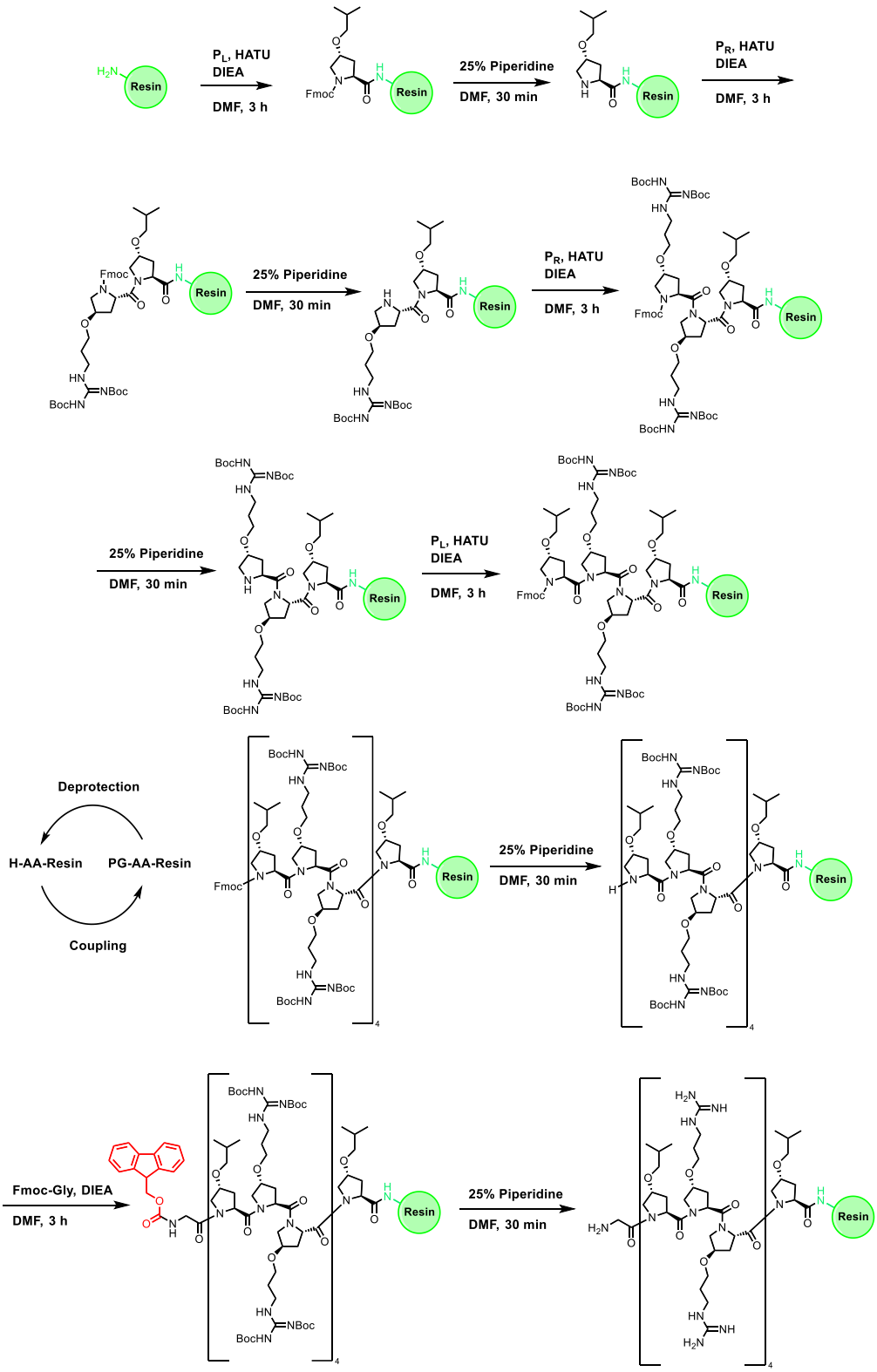
Scheme 3: Synthetic pathway for Fmoc-P_R.

The literature procedure was also used to synthesize the hydrophobic amino acid P_L (Scheme 4).[9] Cbz-hydroxyproline was treated with NaH and 3-bromo-2-methylpropene to provide **3**. This is followed by a hydrogenation to reduce the double bond and remove the Cbz protecting group, followed by reaction with Fmoc-OSu to provide P_L. The overall percent yield of P_L was as expected at 53%, compared to the 60% yield reported in literature.[9]



Scheme 4: Synthetic pathway for Fmoc-P_L.

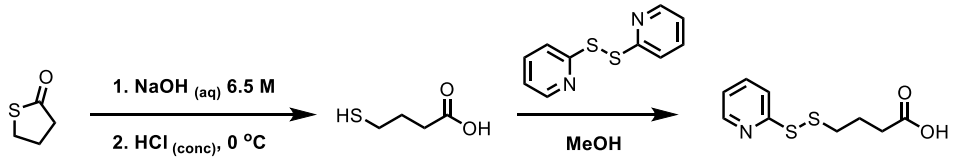
Once the unnatural amino acids were in hand, solid phase peptide synthesis began and followed the steps presented in Scheme 4. P_L was the first amino acid to be coupled onto the H-Rink Amide Resin and this reaction was carried out with HATU and DIEA in DMF for 3 hours. Next, a 25% piperidine solution in DMF with a duration of 30 minutes provided the deprotection of the Fmoc group, which allowed for coupling of the next unnatural amino acid, P_R. The cycle of coupling/deprotection was repeated until the desired peptide was achieved.



Scheme 5: Synthetic pathway for solid phase peptide synthesis of resin-bound P14LRR.

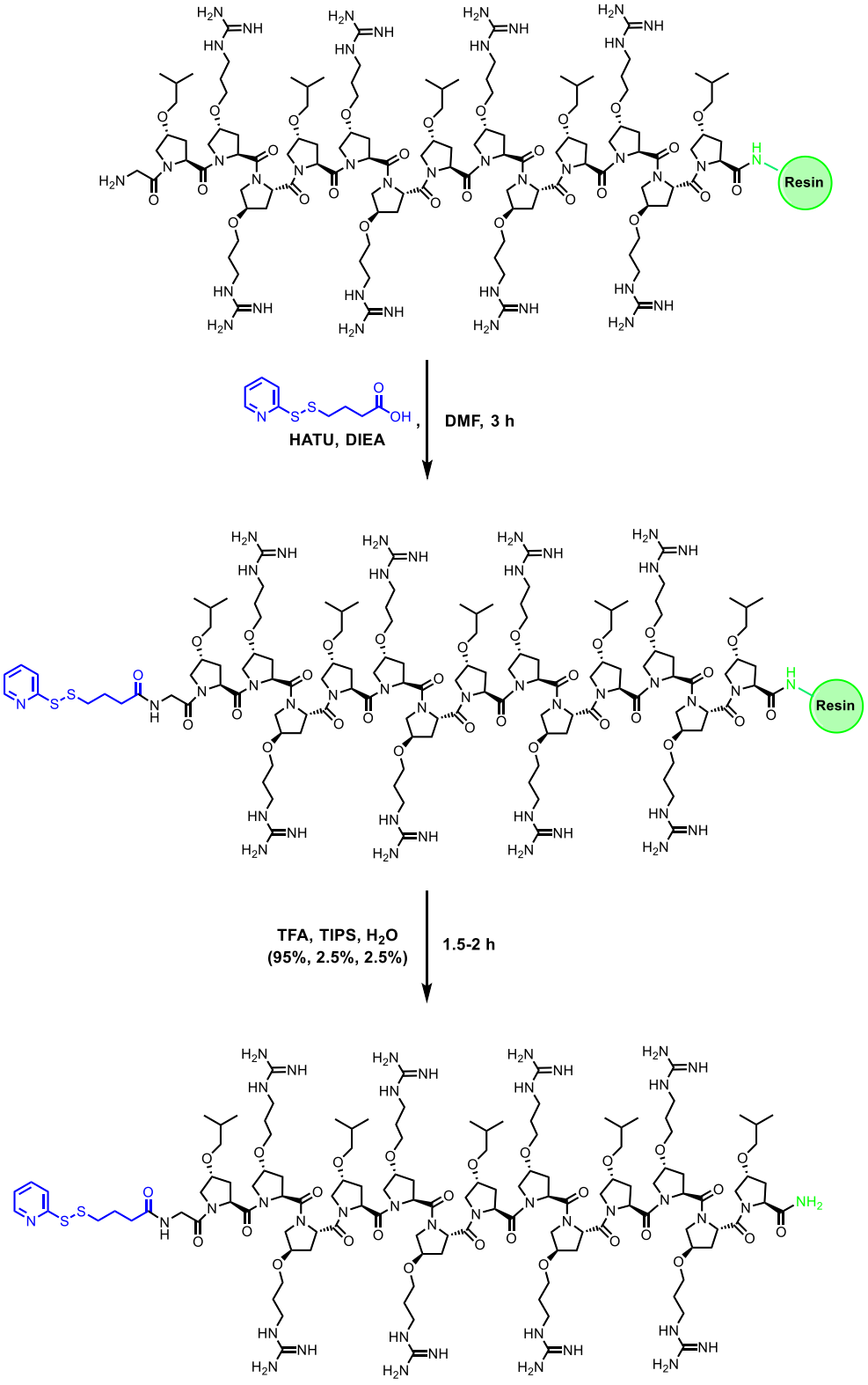
1.2.3 Attachment of the Disulfide Tether to the Peptide

The disulfide tether was synthesized in two steps from a literature procedure (Scheme 6). First the thiolactone underwent a ring opening with NaOH to provide 4-mercaptobutanoic acid. 4-mercaptobutanoic acid was then reacted with 2,2'-dithiopyridine in MeOH, to provide 2-mercaptopyridinyl-4-mercaptobutyric acid disulfide.



Scheme 6: Two step synthesis of the disulfide tether.

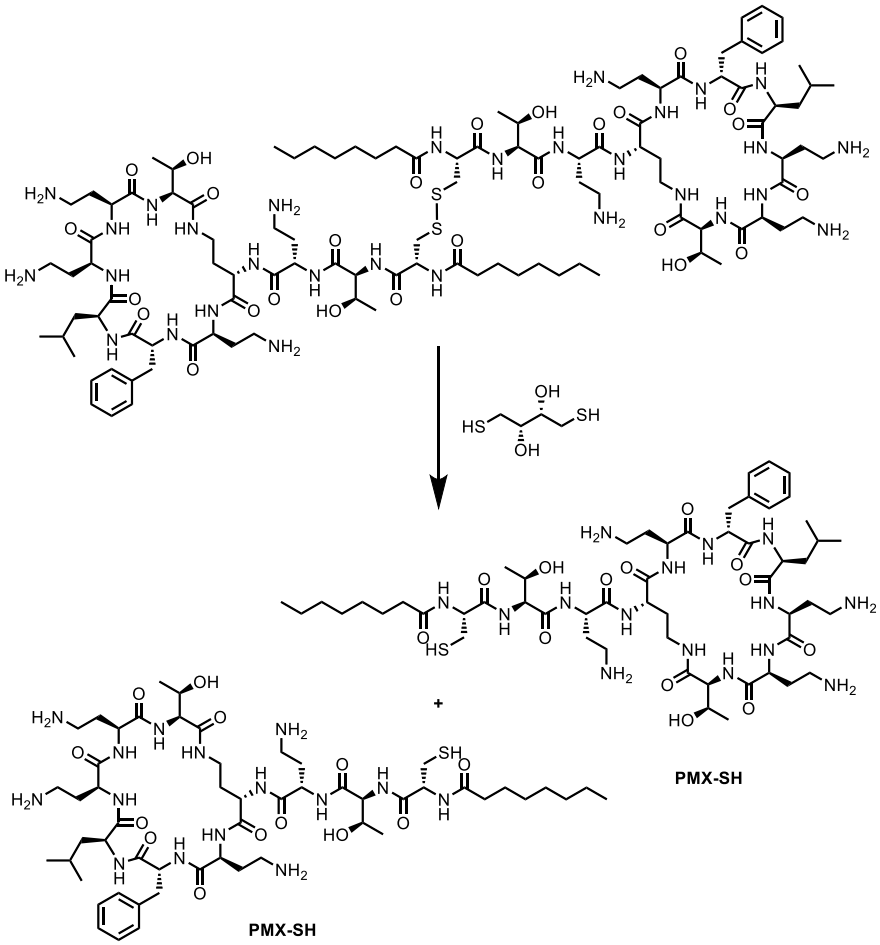
To provide P14LRRSSPy, the Fmoc protecting group of P14LRR on resin was deprotected using a solution of 25% piperidine in DMF for 30 minutes (Scheme 5). Next, the tether was coupled onto the glycine using HATU and DIEA in DMF (Scheme 7). Cleavage of the peptide from the resin was then achieved by spinning in a cocktail of TFA, TIPS and H_2O (95%, 2.5% and 2.5%, respectively). Lastly, the peptide was purified by RP-HPLC.



Scheme 7: Synthetic pathway for solid phase peptide synthesis of P14LRRSSPy.

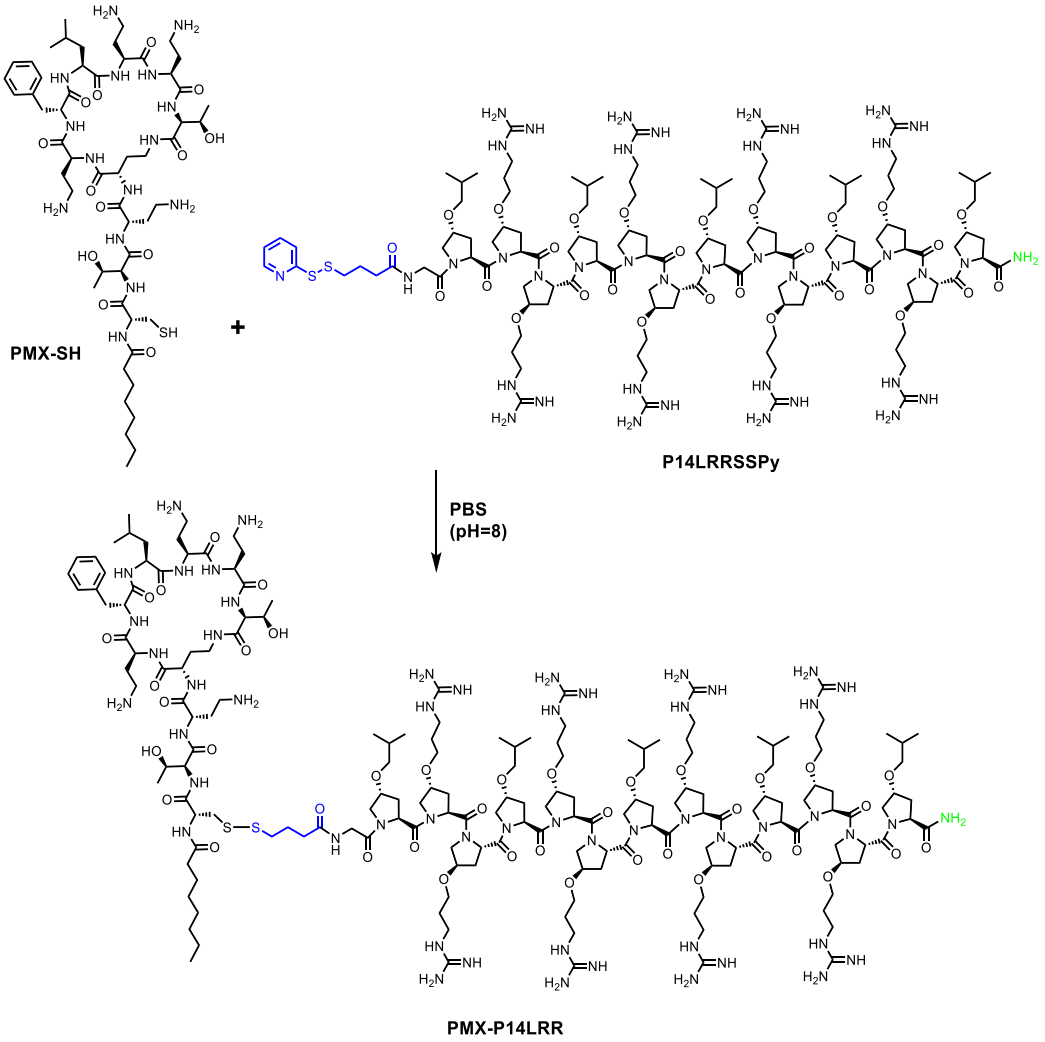
1.2.4 Conjugation of PMX-SH to the Thiol-Modified Peptide

Due to PMX-SH originally being in the dimeric form, it required reduction of the disulfide bond before it could be utilized in the conjugation reaction (Scheme 8). PMX-SH dimer was reduced using a DTT-column (Thermo Scientific), and MALDI-TOF analysis of the fractions confirmed the presence of PMX-SH monomer and dimer.



Scheme 8: Reduction of PMX-SH dimer with DTT to produce two equivalents of the monomer.

The PMX-SH fractions were treated with P14LRRSSPy in PBS buffer (pH 8) (Scheme 9) to provide the dual antibiotic conjugate P14LRR-PMX.



Scheme 9: Formation of the dual antibiotic by an SN2 reaction, which releases 2-mercaptopyridine.

Once the reaction was stopped at the 24-hour mark, the mixture was evaluated for separation by analytical HPLC. The optimized conditions were used to obtain pure P14LRR-PMX and recovered P14LRRSSPy (0.14 mg and 0.83 mg, respectively). Subsequent MALDI mass spectrometry analysis confirmed the synthesis of the conjugate (Figure A6 and Figure A7).

1.3 Conclusions

Conjugates composed of antimicrobial agents can provide dual bacteria-clearing activity with synergistic mechanisms. Alone, the broad-spectrum antibiotic P14LRR has shown the ability to eradicate pathogens and penetrate cell membranes while not lysing them. When linked to another agent such as the aminoglycoside kanamycin, it served these functions while also acting as a delivery vehicle into macrophage cells. With the P14LRR-PMX dual antibiotic, similar activity, but different modes of action are expected. The conjugate has the potential to be broad spectrum and display high cellular accumulation and improved antimicrobial activity while not lysing membranes. However before these properties can be investigated, a higher yield of P14LRR-PMX is needed, for all future experiments. Antibiotic resistance will continue to be a significant problem, but as long as we continue to find ways to bring back antibiotics that pathogens have become resistant to and introduce new agents, we can be a few steps ahead of these bacterial threats.

1.4 Future Directions

Since the conjugation reaction between P14LRR and PMX was successful, the next step would be to carry out this reaction with improved yields of the dual agent P14LRR-PMX. Additionally, a conjugation reaction should be performed to produce a fluorescent analog of P14LRR-PMX for confocal imaging in order to compare the subcellular localization of P14LRR-PMX to the already established P14LRR. Cell uptake and subcellular localization studies in J774A.1 cells would be the next step in determining how well the conjugate accumulates and where it may perform antimicrobial functions within the cells. Lastly, *in cyto* and *in vivo* cell culture work with different bacterial strains would be performed.

1.5 Material and Methods

1.5.1 Materials

The starting material for the unnatural amino acids, Cbz-L-trans-4-hydroxyproline, was purchased from Chem-Impex Inc (Wood Dale, IL, USA), while all other required chemicals were purchased from AK Scientific (Union City, CA, USA) as well as Sigma Aldrich (St. Louis, MO, USA). The polymyxin dimer and monomer were provided by the Pires group at the University of

Virginia (Charlottesville, Virginia, USA) and the Pierce Immobilized Reductant Column was purchased from Thermo Fisher Scientific (Waltham, MA, USA). For solid phase peptide synthesis, the ChemMatrix Rink Amide Resin was purchased from PCAS BioMatrix Inc (Saint-Jean-sur-Richelieu, Quebec, Canada). The Fmoc-Gly and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) were purchased from Ana Spec Inc (Fremont, CA, USA). For the reductant column, the 1,4-dithiothreitol (DTT) was purchased from Roche. N,N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) were all purchased from Sigma Aldrich (St. Louis, MO, USA).

1.5.2 Methods

1.5.2.1 P_R and P_L Synthesis

To produce P_R, three reactions were performed. In the first reaction, two round bottomed flasks (100 and 250 mL) were first equipped with 4 Å molecular sieves and stir bars. The flasks were flame dried and rubber septa were immediately placed. In a hood, the flasks were clamped, and nitrogen was allowed to flow into the flasks through two needles. This setup was used to fill two balloons with nitrogen and the needles from the hood were removed and replaced with the nitrogen balloons for constant flow of nitrogen within the flasks. Ice baths were placed under each flask and THF was added to each flask (20 mL). In the hood a nitrogen needle was inserted into the septa to purge the smaller flask as Cbz-hydroxyproline (2.00 g, 1 mmol) was added using an oven dried spatula. After this addition, the nitrogen needle was removed, and the purging step was repeated for the second flask as NaH (60% Dispersion in Mineral Oil) (1.05 g, 3.5 mmol) was carefully added using an oven dried spatula (aluminum foil was used to cover the ice bath and MeOH and sand were at close proximity in case of fire). A cannula was used to transfer the Cbz-hydroxyproline dropwise into the flask containing the NaH. After an hour of stirring, acrylonitrile (1.97 mL, 4 mmol) was added by syringe, the flask was removed from ice and allowed to stir at room temperature for 24 hours. Some of the solution was placed in a vial and diluted with ethyl acetate and a few drops of 10% HCl. TLC analysis was done using DCM, MeOH and AcOH (90%, 9% and 1%, respectively). For the work up, water was added dropwise to the flask in order to quench the NaH and the THF was removed by rotary evaporator. After this, 10% HCl was used to

reach a pH of 1 and the solution was vacuum filtered to remove the sieves. An extraction was done using water and ethyl acetate and the organic layer was dried with magnesium sulfate before being filtered, rotary evaporated (with toluene washes to remove the acetic acid) and dried on the high vacuum. For purification, flash column chromatography was done using DCM, MeOH and AcOH (95%, 4% and 1%, respectively) and the product **1** was dried on the high vacuum.

For the second reaction, ethanol (20 mL) was added to a round bottom flask containing **1** (530 mg, 1 mmol) and a stir bar. A rubber septum was placed and the flask was purged with nitrogen as palladium on carbon (10% mass of **1**) was added carefully. Two balloons filled with hydrogen were inserted into the septa of the flask and the reaction was left stirring for 24 hours at room temperature. TLC and ninhydrin stain were used to monitor the reaction progress. The reaction was gravity filtered and the ethanol was removed by rotary evaporator. Once a new stir bar and deionized water (10 mL) had been added to the flask, an ice bath was placed under the round bottom flask and sodium bicarbonate was added (420 mg mg, 3 mmol) with stirring. In a falcon tube Fmoc-OSu (618 mg, 1.1 mmol) was dissolved in acetone (10 mL) and this solution was subsequently added dropwise to the mixture in the round bottom flask. The ice bath was removed, and the reaction was allowed to stir overnight at room temperature. TLC and ninhydrin stain were once again used to confirm the consumption of the intermediate and formation of the product. For the work up, 10% HCl was added to achieve a pH<2. Next extraction with ethyl acetate and drying with Na_2SO_4 afforded the product in the organic layer, where the solvent was removed by rotary evaporator and the product **2** was dried in the high vacuum. Flash column chromatography was used to purify the product with a DCM, MeOH and AcOH (96%, 3% and 1%, respectively) isocratic eluent gradient. Purity was confirmed by TLC using DCM, MeOH and AcOH (90%, 9% and 1%, respectively).

In the final reaction in the synthesis of P_R, methanol (20 mL) was added to a round bottom flask containing **2** (870 mg) and a stir bar, followed by acetic acid (1 mL). Next platinum oxide (10% mass of **2**) was added carefully as the flask was purged with nitrogen. Two balloons filled with hydrogen were inserted into the septa of the reaction flask and the reaction was left stirring overnight at room temperature. TLC and ninhydrin stain were used to monitor the reaction progress. A bed of celite was used to filter the reaction and the rotary evaporator was used to remove the methanol. The excess acetic acid was removed using toluene washes. Next a new stir bar was introduced to the flask along with DCM to solubilize the intermediate, which is not very

soluble. The reaction was placed in an ice bath and in a falcon tube triethylamine (895 uL, 3 mmol) was dissolved in DCM (5 mL) and added dropwise into the round bottom. After addition of *N,N'*-Di-Boc-1*H*-pyrazole-1-carboxamidine (866 mg, 1.2 mmol), the flask was taken off the ice and left to stir at room temperature overnight. TLC and ninhydrin stain confirmed the consumption of the intermediate and formation of the product. In order to work up the reaction, more DCM was added to dilute the contents of the flask and washes of the product were done using a saturated sodium bicarbonate solution. After drying with Na2SO4 and removing the solvent by rotary evaporator, flash column chromatography with DCM, MeOH and AcOH (96%, 3% and 1%, respectively) isocratic eluent gradient enabled the purification of P_R. The solvent composed of DCM, MeOH and AcOH (90%, 9% and 1%, respectively) was used to confirm purity by TLC.

Of the two reactions that afford P_L, the first is very similar to the procedure which produces **1**. To start, two round bottomed flasks (100 and 250 mL) were equipped with 4 Å molecular sieves and stir bars, flame dried, and rubber septa were immediately placed. The flasks were clamped in a hood, and purged with nitrogen though two needles. Two balloons with nitrogen and the needles from the hood were removed and replaced with the nitrogen balloons. THF was added to each flask (20 mL) as well as ice baths, where the large flask was in a dry ice bath. Nitrogen was used to purge the flask through a needle in the septa, while Cbz-hydroxyproline (1.00 g, 1 mmol) was added using an oven dried spatula. After the addition, the nitrogen needle was removed. Purging with nitrogen was repeated as NaH (60% Dispersion in Mineral Oil) (530 mg, 3.5 mmol) was carefully added to the second flask using an oven dried spatula (once again aluminum foil was used to cover the ice bath and MeOH and sand were in reach in case of fire). A cannula was used to transfer the Cbz-hydroxyproline dropwise into the larger flask containing the NaH. After 30 minutes of stirring, 3-bromo-2-methylpropene (1.52 mL, 4 mmol) was added using a syringe and following a period of 30 more minutes of stirring, the dry ice bath was removed and stirring resumed at room temperature overnight. Some of the solution was placed in a vial and a few drops of 10% HCl were added to reach a pH of 1. TLC analysis was done using DCM, MeOH and AcOH (93%, 6% and 1%, respectively). For the work up, water was added dropwise to the flask and the THF was rotary evaporated. After this, 10% HCl was used to reach a pH of 1 and vacuum filtering removed the sieves. Extraction using water and DCM provided the product in the organic layer, which was dried with Na_2SO_4 before being filtered, rotary evaporated (including toluene washes to remove the acetic acid) and dried on the high vacuum. For purification, flash column

chromatography was performed using DCM, MeOH and AcOH (93%, 6% and 1%, respectively) and the product **3** was dried on the high vacuum.

The final reaction to achieve P_L began with the addition of methanol (20 mL) to a round bottom flask containing **3** (570 mg, 1 mmol) and a stir bar. A rubber septum was placed on the flask. Subsequently, the flask was purged with nitrogen while palladium on carbon (10% mass of **3**) was added carefully to it. Two pre-filled hydrogen balloons were inserted into the septa of the flask and the reaction was left stirring at room temperature overnight. TLC and ninhydrin stain were used to monitor the consumption of the starting material. The reaction was gravity filtered with two pieces of filter paper and the methanol was removed by rotary evaporator. A new stir bar was added along with deionized water (10 mL) to solubilize the intermediate. The reaction was placed in an ice bath and sodium bicarbonate was added (450 mg, 3 mmol) with stirring. In a falcon tube Fmoc-OSu (662 mg, 1.1 mmol) was solubilized in acetone (10 mL) and the solution was added dropwise to the mixture in the round bottom flask. The ice bath was removed, and the reaction was left to stir overnight at room temperature. To evaluate intermediate consumption and product formation TLC and ninhydrin stain were used. The reaction was acidified with 10% HCl to reach a pH<2. Extraction was done with ethyl acetate and then with saturated sodium chloride. The organic layer was dried with Na_2SO_4 to afford the product in solvent, which was removed by rotary evaporator before drying in the high vacuum. Flash column chromatography was used to purify the product in DCM, MeOH and AcOH (97%, 2% and 1%, respectively) and purity was confirmed by TLC. ^{1}H NMR analysis confirmed the formation of the desired products as peaks in the spectra corresponded to expected proton chemical shifts in the structure.

1.5.2.2 Characterization of Unnatural Amino Acids

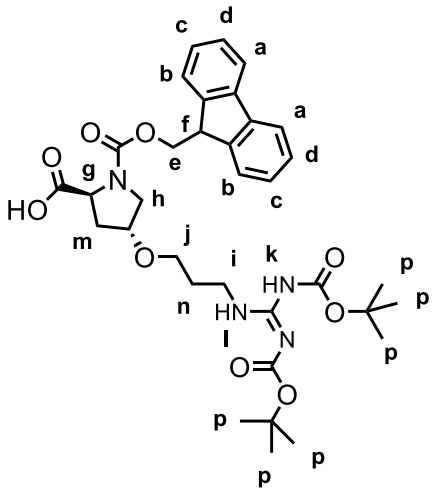


Figure 3: Structure of Fmoc-P_R.

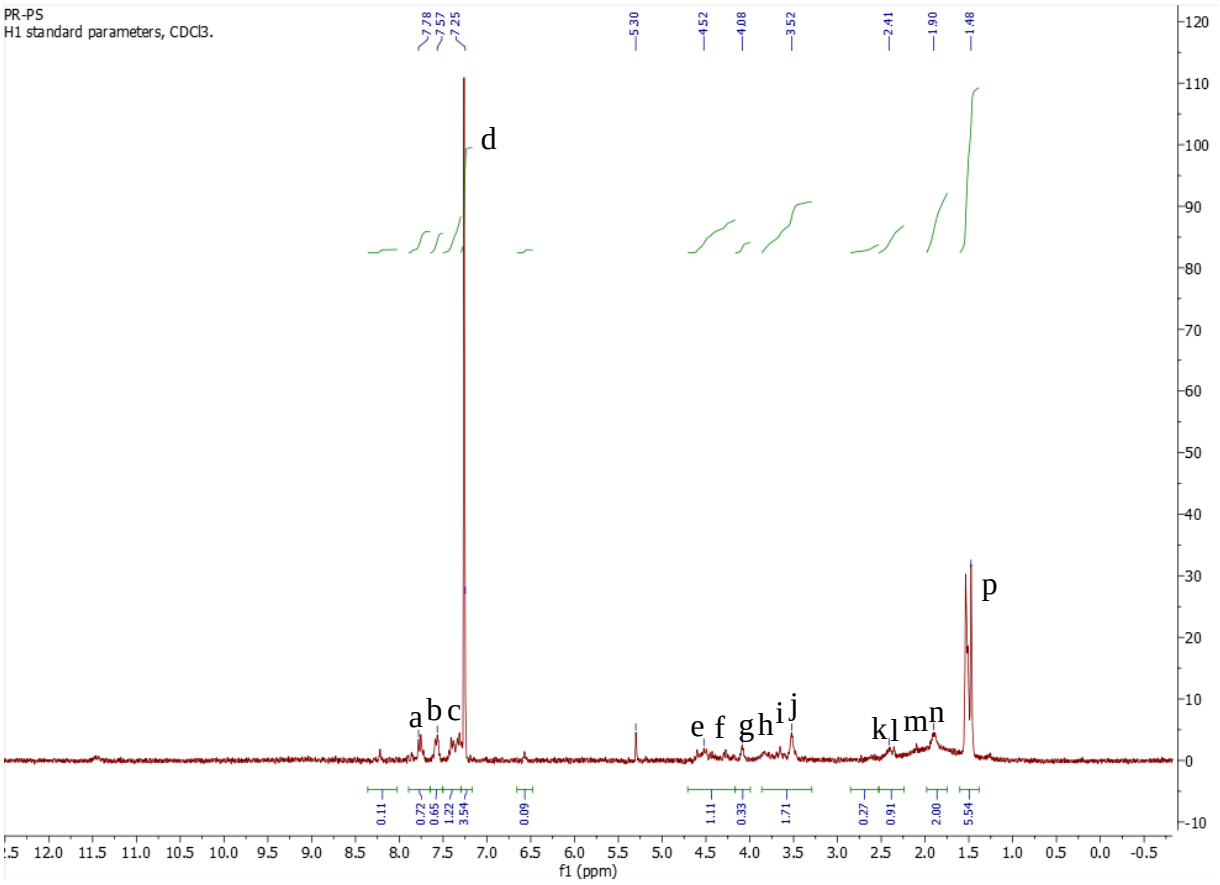


Figure 4: ^{1}H NMR spectrum of Fmoc-P_R.

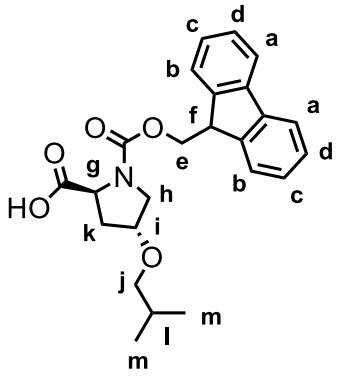


Figure 5: Structure of Fmoc-P_L.

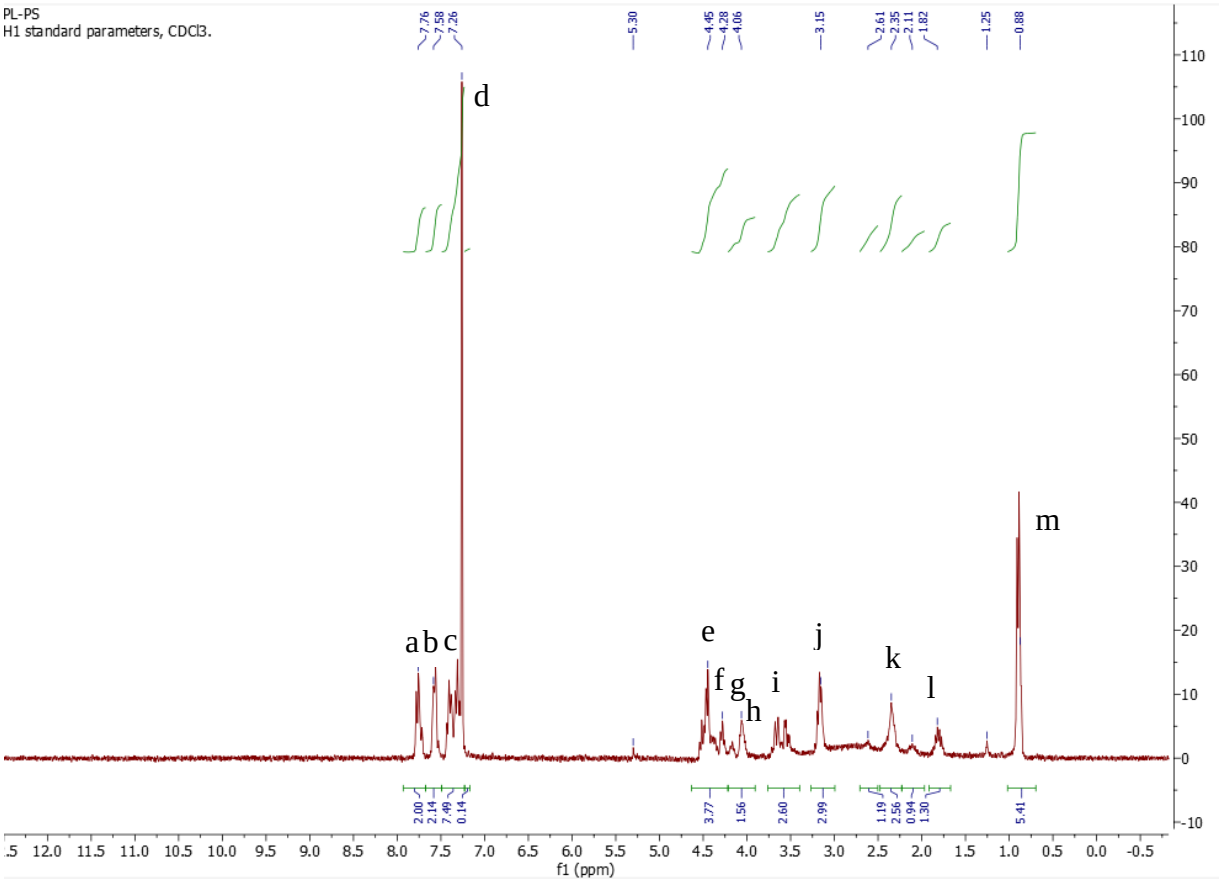


Figure 6: ^{1}H NMR spectrum of Fmoc-P_L.

1.5.2.3 P14LRRSSPy Synthesis

The peptide P14LRR on resin was synthesized using high loading Chem Matrix H-Rink Amide Resin (150 mg, 0.45 mmol/g) through solid phase peptide synthesis. Once the resin was added to the peptide synthesis flask, Fmoc-P_L (55.3 mg, 2 mmol eq), Fmoc-P_R (88.1 mg, 2 mmol) or Fmoc-glycine (80.0 mg, 4 mmol) were added followed by HATU (51.3 mg, 2 mmol) and diisopropylethylamine (46.9 uL, 4 mmol) in DMF (5 mL). If needed, more DMF was added to fill reaction flask at least halfway. A rotary spinner was used to spin the flask for 3 hours at room temperature. Once the coupling time was complete, the flask was drained and washed with DMF, DCM, MeOH and DCM (5 mL, 2X each). Following the coupling of the first Fmoc-P_L, a Kaiser test was used to determine the success of the reaction. Deprotection of the Fmoc group was achieved by adding 25% piperidine in DMF (5 mL) to the peptide synthesis flask and spinning for 30 minutes at room temperature. After this time, the flask was drained and the resin was washed with DMF, DCM, MeOH and DCM (5 mL, 2X each). The success of the deprotection and coupling was tested using a Chloranil test or Kaiser test, as were all subsequent couplings/deprotections. These steps were repeated until all amino acids were coupled onto the resin, then the final Fmoc protecting group was removed.

The peptide P14LRRSSPy was synthesized from the resin bound P14LRR. Finally, 2-mercaptopyridinyl-4-mercaptobutyric acid disulfide (31.0 mg, 4 mmol) was added followed by HATU (51.3 mg, 4 mmol) and diisopropylethylamine (47.0 uL, 8 mmol) in DMF (5 mL) which furnished P14LRRSSPy on resin.

In order to cleave the peptide from the resin and deprotect it, a cleavage cocktail composed of 95:2.5:2.5 TFA:TIPS:H_2O (15 mL) was made and 5 mL of this solution were added to the peptide synthesis flask which was spun for 3 hours. The flask was drained into a tared falcon tube and the resin was washed using the cocktail again (5 mL, 2X). The last washes of the resin were done with DCM (5 mL, 2X). Purification of the peptide, was carried out using RP-HPLC. The solvents used were acetonitrile with 0.1% TFA (solvent A) and water with 0.1% TFA (solvent B). A C18 column was also used. Through analytical HPLC runs, the gradient that afforded the best separation was 20-70% for P14LRRSSPy. Solvent A was used for the semipreparative HPLC runs at a flow rate of 12 mL/min over the course of 60 minutes. For visualization of the products, UV at 214 nm and 254 nm was used, which assisted in the collection of the peptide as it eluted. The fractions of the desired peptide were combined, and the acetonitrile was evaporated. The remaining

water was then lyophilized. The overall purity of the peptide after purification was visualized through an analytical HPLC. MALDI m/z analysis confirmed the mass of P14LRRSSPy to be as expected (Figure A3).

1.5.2.4 DTT Column

Before using the column, a 1 mM DTT activation solution was prepared in a conical tube by dissolving 1,4-dithiothreitol (15 mg) in filtered deionized water (10 mL). More deionized water was then filtered (50 mL) using a 0.2 micron filter, to be utilized for column washes. The tab at the end of the column was twisted off and the column was clamped in the upright position, draining storage solution into an appropriately labeled empty conical tube. The column was washed with the previously filtered deionized water (10 mL) and it was allowed to drain. Next, the column was activated using the 1 mM activation solution (10 mL), which was allowed to drain through before the column was rinsed with more water (10 mL) and drained. The PMX-SH dimer was first dissolved in filtered deionized water (500 uL) before it was added to the column and followed by filtered deionized water washes (10 mL 3X) which were collected into 1.5 mL microcentrifuge tubes, 500 uL were collected in each tube. MALDI m/z analysis was used to determine which tubes had monomer, dimer and which contained both. The corresponding tubes were then combined and lyophilized before the conjugation reaction was performed. For storage, the column was filled with more water, capped, and placed in the refrigerator.

1.5.2.5 P14LRR-PMX Synthesis

In an Eppendorf, degassed PBS (250 uL, pH=8) and P14LRRSSPy (2.80 mg) were combined. This solution was added dropwise to a separate Eppendorf containing PMX-SH monomer (2.36 mg) and a stir bar. The reaction was left stirring for 24 hours at room temperature. Filtered deionized water (3 mL) was added to the solution and it was filtered using a 0.2 micron filter before being purified by RP-HPLC. The HPLC utilized a C18 column as well as the following solvents: acetonitrile with 0.1% TFA (solvent A) and water with 0.1% TFA (solvent B). The gradient that produced the most efficient separation through an analytical HPLC run was 20-60% solvent A and it was therefore used for the semipreparative HPLC run at a flow rate of 12 mL/min over the course of 60 minutes. UV at 214 nm and 254 nm was used to visualize the products, where

elution of the P14LRR-PMX conjugate occurred at 30.9 mins and elution of P14LRRSSPy occurred at 29.1 mins. The fractions of each respective product were obtained, and the acetonitrile was evaporated, with the remaining water being lyophilized. The weights of the collected products after purification were 0.14 mg for P14LRR-PMX and 0.83 mg for P14LRRSSPy. Both were 99% pure when evaluated by analytical HPLC. MALDI m/z analysis: expected: 3910.85, observed: 3909.6 (P14LRR-PMX) and 2828.9 (P14LRRSSPy), (Figure A6, Figure A7).

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APPENDIX

Purity Analytical

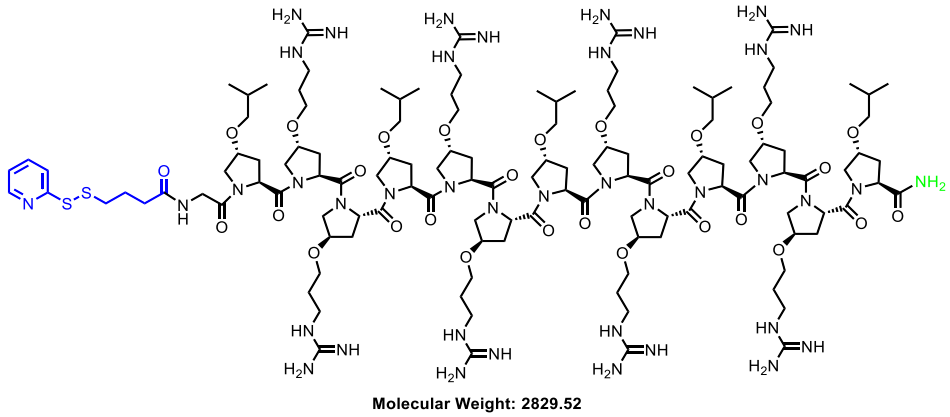

Figure A 1: Structure of P14LRRSSPy.

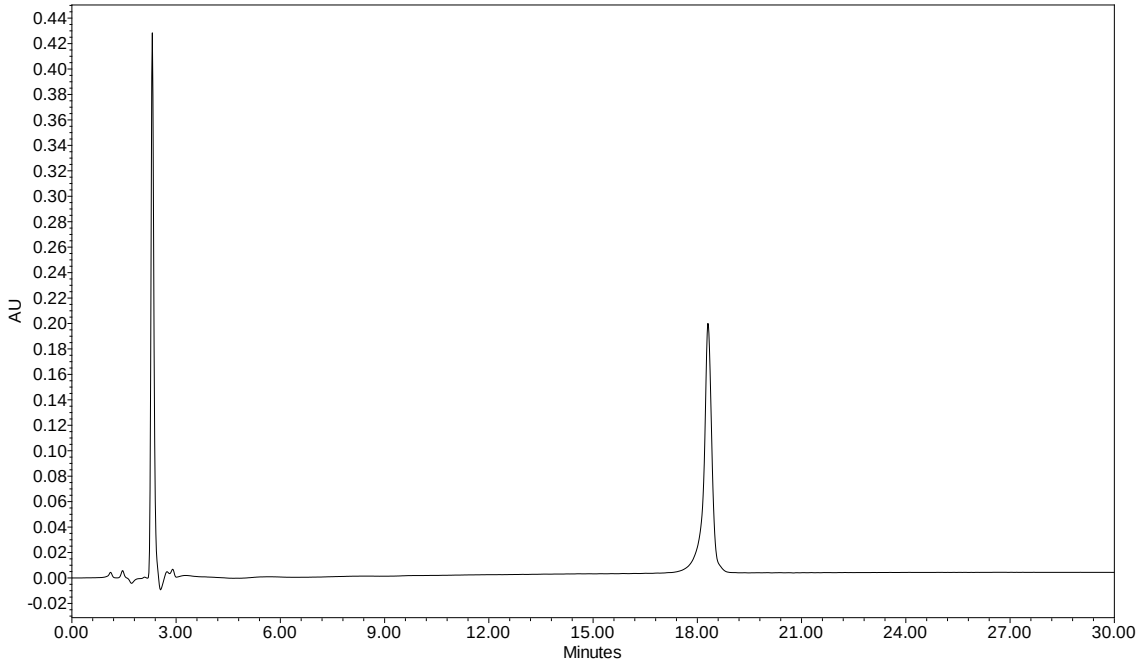

Figure A 2: RP-HPLC analytical chromatogram for P14LRRSSPy.

MALDI

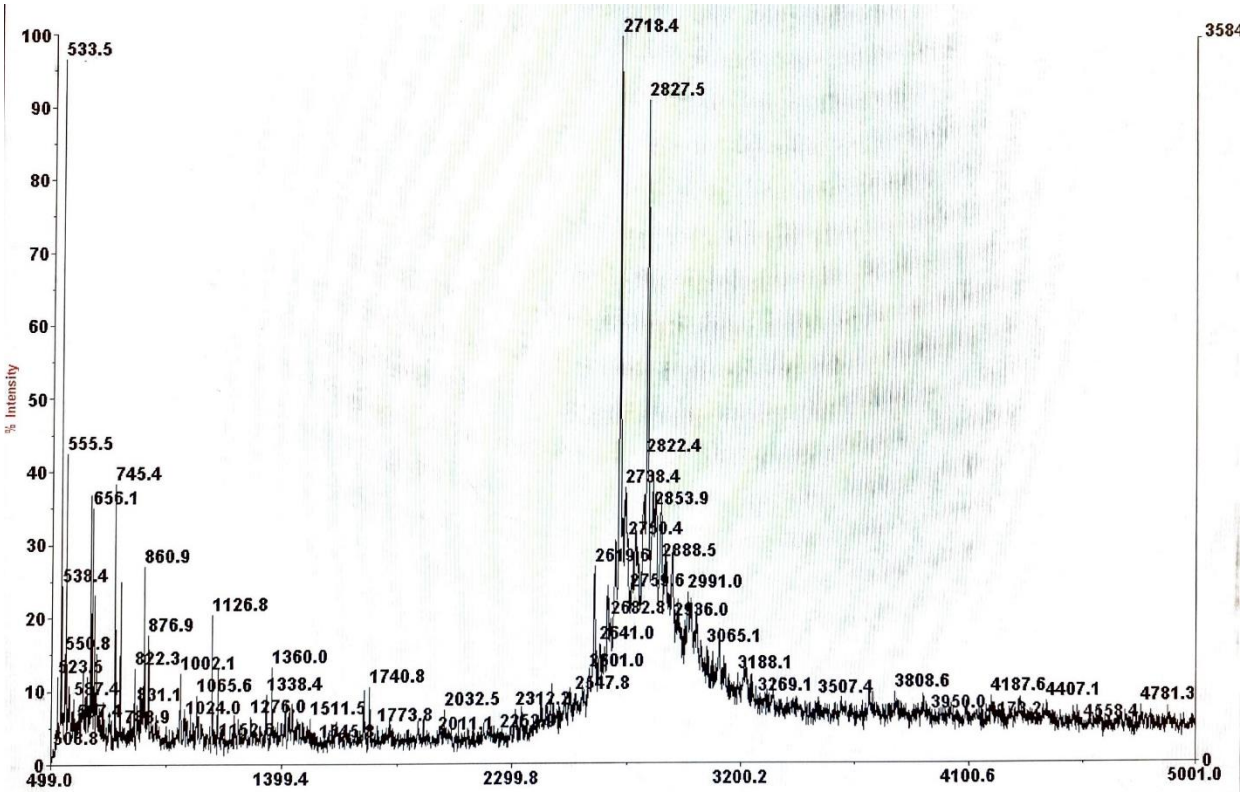

Figure A 3: MALDI mass spectra of P14LRRSSPy (Expected Mass: 2829.5, Observed Mass: 2827.5, 2718.4).

Purity Analytical

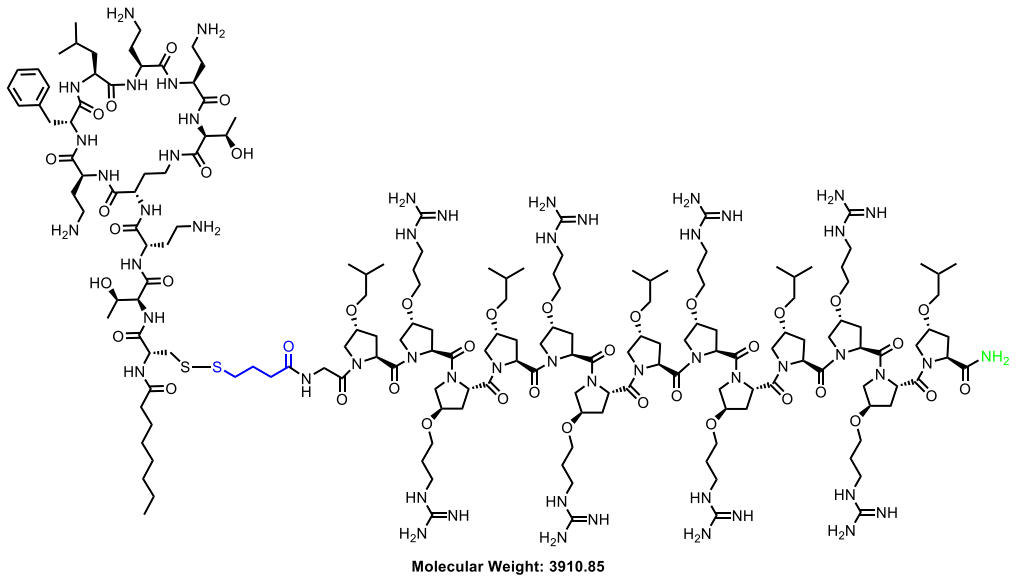

Figure A 4: Structure of P14LRR-PMX.

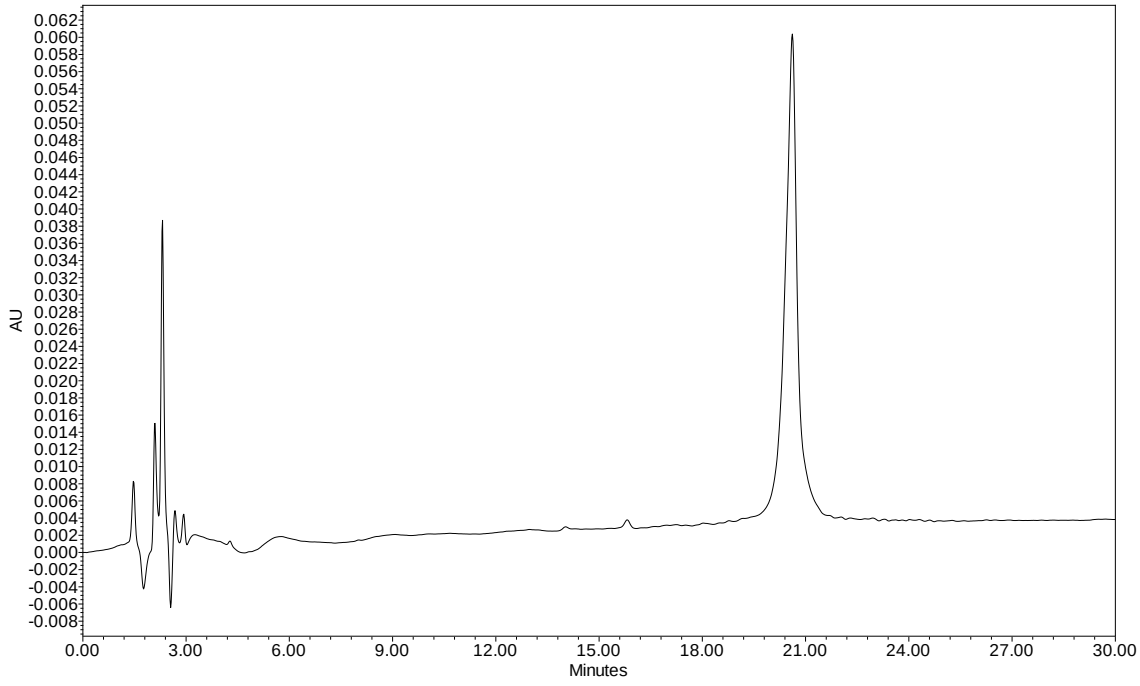

Figure A 5: RP-HPLC analytical chromatogram for P14LRR-PMX.

MALDI

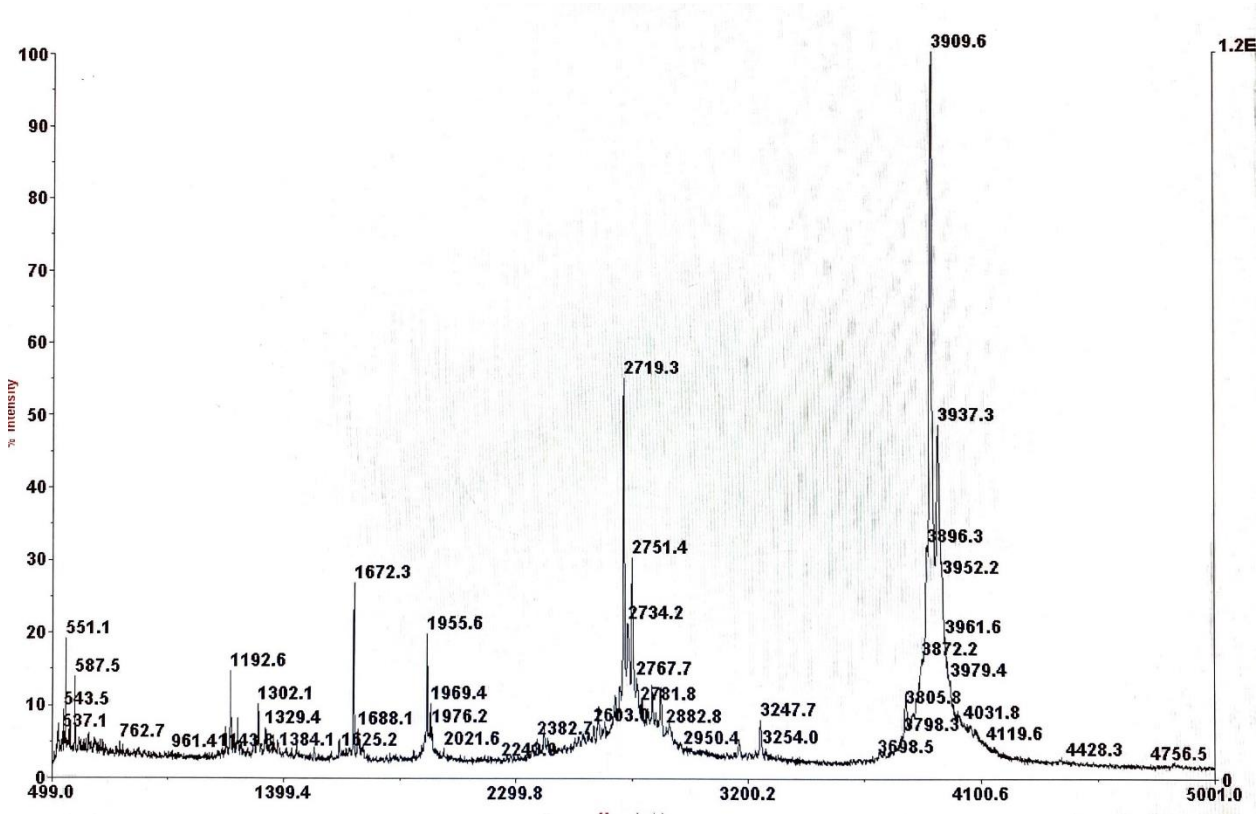

Figure A 6: MALDI mass spectra of P14LRR-PMX (Expected Mass: 3910.8, Observed Mass: 3909.6).

MALDI

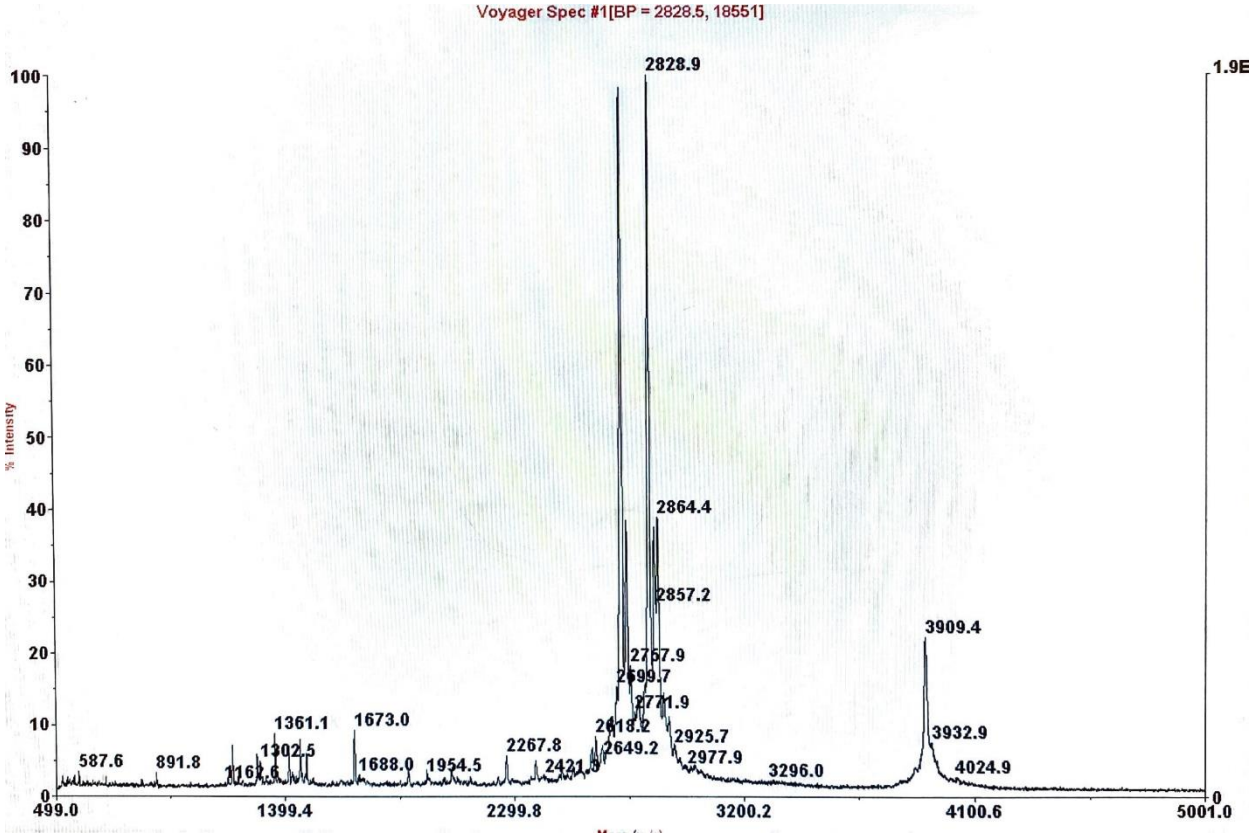

Figure A 7: MALDI mass spectra of P14LRRSSPy (Expected Mass: 2829.5, Observed Mass: 2828.9).