Synthesis of Amphiphilic α- and γ-AApeptides for Antimicrobial, Self-Assembly, and Mineralization Studies

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Synthesis of Amphiphilic $\alpha$- and $\gamma$-$\alpha$Apeptides for Antimicrobial, Self-Assembly, and Mineralization Studies

by

Mohamad Nassir Amin

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science Department of Chemistry College of Arts and Sciences University of South Florida

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Keywords: Peptidomimetics, amphiphiles, biomineralization, antimicrobial peptides, calcium carbonate mineralization

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Dedication

To my parents, for their enormous support. To Hanna for her amazing heart. To my brothers for their everlasting encouragement. To Abid, for his indispensable friendship.

To everyone who was ever there for me when I needed them. To Hadi, for the future.
Acknowledgments

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Abstract

Seven novel, amphiphilic AApeptides were prepared. Two cationic, lipo-α-AApeptides, NA-75 and NA-77 were found to possess potent antimicrobial activity against Gram-positive bacteria, with almost no hemolytic activity. In addition to NA-75 and 77, four amphiphilic, γ-AApeptides, NA-133, 135, 137, and 139, and one anionic lipo-α-AApeptide, NA-81, were prepared for molecular self-assembly studies, with several interesting nanostructures observed by TEM. Mineralization of calcium carbonate from gaseous CO$_2$ and Ca$^{2+}$ in the presence of the 7 AApeptide amphiphiles was also observed by optical microscopy. Several AApeptides were found to be able to influence CaCO$_3$ crystal morphology. Another α-AApeptide, NA-63, was synthesized by a novel, alternative method, which has several potential advantages over the previous synthesis methods.
Chapter 1:

Design and Synthesis of Antimicrobial α-AApeptides

1.1 Introduction

1.1.1 Overview

Peptidomimetics are a diverse class of synthetic compounds designed to mimic the structure and/or function of peptides, but with modified backbones. Unlike natural peptides, peptidomimetics possess backbones which are not based solely on α-L-amino acids. Peptidomimetics are typically designed by modifying peptides, or through mimicry of peptide secondary structure. Peptides are highly involved in important biological processes and are therefore of great pharmaceutical interest.\(^1,2\)

Unfortunately, natural peptides have several disadvantages which may limit their therapeutic application – particularly their susceptibility to proteolysis.\(^3,4\) The development of peptidomimetics, including the newly-developed AApeptides, which are the subject of this research, is an attempt to overcome some of the disadvantages of peptides while retaining their functionality, as well as exploiting any potential advantages offered by the non-natural peptide mimics. Herein we report the novel synthesis of several AApeptides, and subsequent investigation of their potential utility. AApeptides are a new class of peptide mimics developed by our laboratory, derived from the alpha-chiral PNA (peptide-nucleic acid) backbone, shown in Figure 4.\(^3,4\) AApeptides are oligomers of N-acylated-N-aminoethyl amino acids, first reported by members of my
research group in 2011.\textsuperscript{3,4} Our laboratory reported the novel synthesis of both α-AApeptides and γ-AApeptides, which are very similar, but differ in the positioning of side-chains along the backbone.\textsuperscript{3,4}

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{\textbf{α-Peptides, α-AApeptides, and γ-AApeptides.} Representative structure of 12-residue α-peptide (top), 6-block α-AApeptide (middle), and 6-block γ-AApeptide (bottom). AApeptides have the same number of side-chains and amide bonds as α-peptides of the same length.}
\end{figure}

Peptides are of significant biomedical interest, owing to their involvement in practically every vital biological process.\textsuperscript{2} Some functions of peptides that are interesting include disruption of protein-protein interaction, inhibition or modulation of enzyme activity, and (as in this research) antimicrobial activity.\textsuperscript{1} The ability to exploit peptides as potential pharmaceuticals or compounds of other biological interest is highly desirable in bioorganic and chemical biology. Peptidomimetics designed to imitate the structure of interesting peptides may be able to express the functionality of such peptides, or even improve upon them.\textsuperscript{3-5}
A wide variety of peptidomimetics have been reported, mainly differing in their backbone structures. Examples include peptoids, β-peptides, α/β-peptides, γ- and δ- peptides, oligoureas, polyamides, sugar-based peptides, azapeptides, α-aminoxy-peptides, and phenylene ethynlenes. Just as there are a vast number of possible natural peptides, depending on the sequence and number of amino acids, each type of peptidomimetic may represent a vast number of possible compounds, potentially an entire library of compounds whose properties can be further investigated. Therefore, the development of novel peptidomimetic backbones with a high potential for diversification, and straightforward, low-cost synthesis and tunability is greatly needed.

1.1.2 Natural peptides

Natural α peptides represent a vast number of unique compounds, many with pharmaceutically and biologically interesting functions, which are difficult to exploit as pharmaceuticals for several reasons. Natural peptides are susceptible to proteolysis—degradation by proteolytic enzymes. This gives them poor in vivo stability, which limits their use as potential drug candidates. Natural peptides can also be immunogenic, potentially triggering immune response. Such immune responses may further impact the bioavailability of the peptide, or an autoimmune response could be provoked. Nevertheless, many peptides might be quite useful if not for such caveats.

Because peptides are made of chiral, L-α-amino acids, their primary structure is not the only factor in determining their functionality. Peptides, even relatively short ones, adopt a variety of secondary structures, called foldamers, such as α-helices, β-
sheets, etc. These structural motifs are often very important to the peptide's function, especially peptide aptamers. The diverse biological functions of peptides include such physiologically important functions as neurotransmitters, neuromodulators, hormones, as well as many others.1,2

1.1.3 Peptidomimetics

Peptidomimetics have recently begun to be recognized as a potential strategy to modify the structures of interesting peptides in order to make them potentially better as therapeutics or otherwise biomedically useful, especially by improving their biostability and bioavailability. Some peptidomimetics that have been reported are peptoids,6 β-peptides,7-9 α/β-peptides,10,11 γ- and δ- peptides,12-14 oligoureas,15,16 polyamides,17 sugar-based peptides,18,19 azapeptides, α-aminoxy-peptides,20 and phenylene ethynyles.21 D-amino acids may also be considered as falling into this category, since D-amino acids only occur rarely in nature.25-29 Peptidomimetics are often stable to proteolysis, and are believed to possess reduced immunogenicity and improved bioavailability compared to natural peptides.3,4 Certain peptidomimetics have a high potential for diversification, due to the synthetic methods used to make them. Whereas natural peptides are generally limited to the classic 20 natural amino acid side-chains, peptidomimetics may be able to incorporate un-natural side-chains, allowing for greater structural diversity.30,31 The development of novel backbones with new and unique properties is still needed in order to discover new molecules of interest to biomedical and chemical biology.22

Covalently modified peptides occur in nature, often resulting from post-translational modifications. These include lipopeptides, glycopeptides, N- and O-
acylation, etc.$^{32}$ Likewise, peptidomimetics may also be further derivatized by chemical modification. Such possible derivatization strategies for these include cyclization, lipidation, oligosaccharide conjugation, acylation, etc.

As mentioned previously, the primary structure of peptides is not the only factor in their activity, their 3D structure is important too. The spatial orientation of side-chain groups may be equally as important as the composition of the different side-chains themselves.$^3$ Therefore, it is useful for peptidomimetics to be able to display conformational characteristics similar to peptide secondary structure. For example, a helical domain of the tumor suppressor protein p53 binds to MDM2.$^{3,4}$ Disruption of this protein-protein interaction is desirable to anti-cancer research, but challenging to achieve with peptidomimetics, due to the importance of 3D structural orientation of the interacting residues.$^{3,4}$ Although the development of peptidomimetics with well-controlled secondary structure tends not to be straightforward, peptidomimetics which

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<td>γ-AApeptide</td>
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<tr>
<td>β3-peptide</td>
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<tr>
<td>peptoid</td>
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Figure 2: α-Peptides, α-AApeptides, γ-AApeptides, peptoids,$^6$ and β-peptides$^7$–$^9$. 
demonstrate such highly specific interactions have been reported, including AApeptides.3,4

Peptidomimetics may be useful for disruption of protein-protein interactions, inhibition or modulation of enzyme activity, and (as in this project) antimicrobial activity.33 They may also be useful for the investigation of the importance of structural characteristics to peptide activity/behavior.34-36 Peptidomimetics can be made to mimic peptide aptamers, with highly specific interactions, or to mimic broad, global features of peptides, such as overall or regional cationic/anionic charge, hydrophobicity, etc.6,37 Peptidomimetics, including AApeptides, can also be made to mimic short sections of larger peptides, to act as aptamers/inhibitors with high specificity and binding affinity. Some of the functions of short peptides that we are interested in mimicking in this research are antimicrobial activity, self-assembly to form nanostructures, and the ability to influence mineral crystallization. These functions appear to be more dependent upon the overall characteristics of the peptide/peptidomimetic, rather than upon the highly specific interactions of aptamers.36,38-40

1.1.4 AApeptides

As previously mentioned, there are two types of AApeptides, which vary in the position of chiral substitution on the backbone. The two types are shown in Figure 3. Both types of AApeptides were first reported by my research group in 2011.3,4 They are non-natural peptide oligomers based upon chiral PNA (peptide-nucleic acid) backbone.3,4 In early work, both α- and γ-AApeptides were found to be highly resistant to enzymatic hydrolysis by chymotrypsin, trypsin, and pronase.3,4 AApeptides are highly diversifiable,
due to the ability to incorporate a wide variety of side-chains by N-acylation, not limited only to the typical amino acid side-chains.\textsuperscript{3,4,41}

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**Figure 3: \(\alpha\)- and \(\gamma\)-AApeptides.**

Each repeating unit is analogous to a dipeptide, possessing two side-chains, and the same backbone length. This means that longer AApeptides will have the same number of side-chains as natural peptides of the same length. They will also have the same number of backbone amide bonds as natural \(\alpha\)-peptides for a given chain length. These structural similarities, along with the improved resistance to proteolysis, and straightforward diversifiability and tunability, should make AApeptides very good candidates for development of new peptide-based biomimetic compounds, such as chemical probes, pharmaceuticals, etc.\textsuperscript{3,4,42,43}

AApeptides have only half the number of chiral centers per side chain as a corresponding \(\alpha\)-peptide. One half of the backbone amides are tertiary amides, which can be oriented either \textit{cis} or \textit{trans}, and no longer possess the same H-bonding characteristics as the secondary amide of an \(\alpha\)-peptide. This means that the secondary structure is expected to be different than that of \(\alpha\)-peptides. The structural properties of AApeptides are still not fully explored yet; further study should improve the ability to predict and control AApeptide secondary structural characteristics.
Since the introduction of AApeptides, there have been multiple strategies used in their synthesis. The first was by synthesizing "dipeptidomimetic" building blocks, each with two side-chain groups (protected as necessary), an Fmoc-protected amino group, and an acid group. These were then linked to other building blocks by solid-phase synthesis, in much the same way as is done for synthetic α-peptides. This method can be used to make both α- and γ-AApeptides, from their corresponding building blocks units.\(^3,4,31\)

Another method was developed and is reported here for the first time, using building blocks with alloc-protection of the terminal nitrogen, and Fmoc-protection of the secondary amine, rather than acylation to introduce another side chain. See Figure 12 for an illustration of this strategy. This would allow the N-acyl side chain to be added during solid-phase synthesis, following deprotection to the secondary amine, by acylation using
any compatible carboxylic acid or acyl chloride. De-alloc-protection can be performed during solid phase synthesis using tetrakis(triphenylphosphine) palladium (0) as a catalyst. This method could allow a greater variety of possible sequences to be made from a given number of building blocks, compared to using the previously established method, since only one of the two side-chains of each building block would be pre-determined. This method has been used to make α-AApeptides, as reported in this research, and a very similar approach was used by our laboratory to make γ-AApeptides by a combinatorial approach.

Notably, it is possible to synthesize γ-AApeptides by a sub-monomer strategy, circumventing building block synthesis, and preparing the sequences entirely on solid phase, from relatively simpler units. This is reported by our laboratory previously, but falls outside the scope of this manuscript.

AApeptides have a high potential for diversification, especially at the tertiary amide. Because the acylation is done using any of a wide variety of commercially
available carboxylic acid or acyl chloride derivatives, the possibilities for half the side-chains are practically limitless. The other side chain typically comes from a natural α-peptide, but other non-natural α-peptides could be incorporated as well, such as D-amino acids, or amino acids with non-natural side-chains, etc.\textsuperscript{3,4}

The first α-AA peptides were reported by our laboratory in 2011.\textsuperscript{3,4} They were found to selectively inhibit protein-protein interaction between p53 and MDM2. They showed high selectivity, and also significant stability to proteolysis.\textsuperscript{3} Soon after, our lab reported γ-AA peptides, which were also able to disrupt p53/MDM2 interaction, with similarly favorable stability toward enzymatic degradation.\textsuperscript{4} This initial work provides evidence for the potential applicability of AApeptides as non-natural oligomers with potent bioactivity, high specificity, and stability.\textsuperscript{3,4}

Although the continued investigation of AApeptide aptamers is important, the focus of this research was on compounds whose overall structural characteristics are
more important to their function than their precise molecular structure. The common feature of the peptidomimetics that were synthesized over the course of this research is that they are amphiphilic, with part of the molecule hydrophilic due to charge in aqueous solution, and part of the molecule hydrophobic. My research has focused on the development of novel synthetic methods to make both $\alpha$-and $\gamma$-AApeptides. Several $\alpha$-AApeptides were made to explore potential applications of AApeptides as potential novel antimicrobial agents, inspired by AMPs.

1.1.5 Antimicrobial peptides

Antimicrobial peptides (AMPs), also called host defense peptides (HDPs), are found in nearly all living organisms, including humans, and play an important role in the innate immune system, providing defense against a variety of infections.\textsuperscript{45} Generally, they are short, cationic, amphiphilic peptides, often adopting helical conformations.\textsuperscript{45,46} Cationicity is due to a large number of basic residues, such as lysine, histidine, tryptophan, or arginine, which are protonated under physiological pH. Hydrophobicity is typically afforded by hydrophobic residues, such as phenylalanine, valine, leucine, and isoleucine. Magainin is a typical example of a helical AMP, while indolicidin is an example of an AMP that adopts an extended structure.\textsuperscript{47} There is a hope that AMPs could lead to a new type of broad-range antibiotic, which provides protection from a variety of pathogenic bacteria, without causing resistance to develop.\textsuperscript{35,47-49}

AMPs have come to be regarded as a potential source of antibiotics for future use, serving as drug leads to guide the development of new types of antibiotics.\textsuperscript{35,49} Current conventional antibiotics have become increasingly ineffective over the years, as many
microbes have developed resistance to them. This problem has very serious global health consequences. It is hoped that AMP-inspired antibiotics could circumvent bacterial resistance, since, unlike most conventional antibiotics, which act through highly specific interactions with bacterial enzymes, proteins, etc., AMPs are believed to act through more general interactions with the bacterial membrane. They have low susceptibility to the development of antibiotic resistance, most importantly.

AMPs' overall cationic charge and amphipathicity allow them to cause disruption of bacterial membranes, by causing membrane thinning, poration, and leakage. The exact mechanism is not precisely known, but it is believed that charge interactions between the cationic AMPs and the anionic bacterial cell membranes cause the AMPs to accumulate on the cells, where hydrophobic interactions with membrane phospholipids cause the AMP to insert into the membrane. This results in a loss of membrane integrity, and microbial death. Since mammalian cell membranes are zwitterionic, there is no electrostatic attraction to the cationic AMPs. This allows AMPs to have high selectivity toward pathogens, without damaging mammalian cells. Because of the membrane-disruption mechanism, acquiring resistance to AMPs is more difficult compared to conventional antibiotics. Indeed, AMPs have been a part of the innate immune defense of organisms for millions of years, yet are still effective.

Conventional antibiotics typically act by highly specific interactions with some bacterial enzyme or protein. This leads to antimicrobial resistance by several mechanisms, including mutations of enzymes so that they are no longer inhibited by the antibiotic, but retain their functionality. Such mechanisms allow bacteria to very quickly develop resistance to new antibiotics. Due to their unique mechanism of action,
AMPs are able to provide protection from a variety of pathogenic bacteria, acting as broad-range antibiotics, without propensity for inducing resistance.\textsuperscript{48}

AMPs have many of the same potential drawbacks as many other biomedically interesting peptides: they are intrinsically unstable to degradation by proteolysis, and they may also be immunogenic.\textsuperscript{33} Also, they typically only have moderate antimicrobial activities, leaving more potent activity to be desired.\textsuperscript{52} Their structure is relatively simple, and their overall characteristics are more important to their function than the specific sequence of amino acids, which makes development of peptidomimetic analogues simpler and more straightforward, compared to peptide aptamers, which tend to require highly specific 3-dimensional positioning. Although many AMPs possess a helical structure, it is believed that this may not be necessary to their antimicrobial activity.\textsuperscript{40,53,54} Rather, helical AMPs may have higher hemolytic activity compared to those with extended or random-coil structure.\textsuperscript{38,49}

Because AMPs have activity against a broad range of antimicrobial organisms, it is hoped that peptidomimetic analogues would have a similar broad-range of activity, leading to new antibiotics for potential therapeutic use. Therefore, a few types of peptidomimetic analogues of AMPs have been reported, such as β-peptides, arylamides, and peptoids.\textsuperscript{31}

The addition of saturated fatty acid tails to AMPs has been shown to greatly improve the bactericidal activity against both Gram-positive and Gram-negative strains.\textsuperscript{55-57} Reports of lipidated peptidomimetics are rare,\textsuperscript{35} but short peptoid AMP mimetics were also shown to bear improved selectivity and retained antimicrobial activity when alkylated with 10-13 carbon lipids. Polymyxin B and Daptomycin are two
antibiotics, which are lipidated peptides, whose fatty acid tails are integral to their antibacterial activity.

1.1.6 Design and synthetic strategy of α-AApeptides

In 2011, the development of unlipidated α- and γ-AApeptides with broad-spectrum antimicrobial activity and low hemolytic activity was reported by others in my group. Following this, we began to develop lipidated AApeptides to further investigate their activity and properties, and with the hypothesis that the addition of a lipid tail to the previous motif of hydrophobic/cationic side-chains might improve either the activity or the selectivity. I synthesized the lipo-α-AApeptides shown in Figure 7 for study. Two sequences, NA-75 and 77, were synthesized on solid support, via the standard α-AApeptide building block method, described in detail in Section 1.2.3 of this chapter.

Figure 7: NA-75 and 77.
The synthesized AApeptides then underwent testing to determine their antimicrobial activity against a series of interesting/pathogenic microbes, including strains with resistance to conventional antibiotics. Hemolysis testing was also done to assess the selectivity against bacterial/fungal cells vs mammalian cells. The development and activity of NA-75 and NA-77 have been reported in a previously published work,\textsuperscript{31} as well as this manuscript. Lipo-\(\alpha\)-AApeptides NA-75 and 77, inspired by AMPs, are similarly short, cationic, amphiphilic oligomers, with cationicity provided by amino side-chains from the lysine residue, and hydrophobicity provided by the hydrophobic N-acyl side-chains and an aliphatic palmitoyl tail added to the terminal N. An overview of the synthetic scheme is given in Figure 11.

To explore alternative synthesis methods, another sequence, NA-63, was made, using alloc/Fmoc protected building blocks, as shown in Figure 12. As a model, a Phe-based alloc/Fmoc \(\alpha\)-AApeptide building block was prepared, and a tri-block sequence with hydrocinnamic acid side-chains added during solid phase synthesis was prepared. This is the first report of an \(\alpha\)-AApeptide made by this method.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{NA-63.png}
\caption{NA-63.}
\end{figure}

1.2 Experimental
1.2.1 α-AApeptide building block synthesis

The previously reported \(^3\) procedure for the synthesis of a representative α-AAapeptide building block (lys/hydrocinnamic acid) is shown below:

Fmoc-Lys (Boc)-OH was benzylated overnight at room temperature in DMF using 5 eq benzyl bromide and 5 eq solid sodium bicarbonate. After purification, Fmoc protection was removed from the benzylated product using 2:1 ratio of acetonitrile:diethylamine. This was followed by reductive amination at 0 °C using 1 eq Fmoc-glycine-aldehyde and 1.7 eq sodium cyanoborohydride in methanol. After purification of the secondary amine, it was subjected to acid coupling overnight at room temperature with hydrocinnamic acid using 5 eq DhbtoH (3-hydroxy-1,2,3-benzotriazin-4(3H)-one) and 5 eq DIC (N, N’-Diisopropylcarbodiimide) as activation agents. The final step was hydrogenation, which was carried out in the presence of H\(_2\) gas and 20% palladium on activated carbon as catalyst in ethanol. The other building block, shown in Figure 9, was prepared similarly, but by acylating the secondary amine with 4-methylvaleric acid instead.

![Figure 9: α-AAapeptide building blocks](image)

**Figure 9: α-AAapeptide building blocks.** The building blocks for NA-75 (left), and NA-77 (right).
1.2.2 Fmoc/alloc α-AApeptide building block synthesis

Alloc-protected glycinealdehyde, prepared previously, was reductively aminated at 0 °C using 1 eq phenylalanine t-butyl ester and 1.7 eq NaCNBH₃ in methanol. After purification of the secondary amine, it was Fmoc-protected using 2 eq Fmoc-Cl, overnight, in a mixture of 1:2 CH₂Cl₂: saturated NaHCO₃ in water. The t-butyl ester was deprotected using 20% TFA in CH₂Cl₂, giving the final building block.

![Figure 10: Phenylalanine-based alloc/Fmoc-α-AApeptide building block.](image)

1.2.3 Solid phase synthesis of NA-75 and 77

Lipidated α-AApeptides were synthesized on Rink amide resin in peptide synthesis vessels on a Burrell wrist-action shaker following the standard Fmoc chemistry solid phase peptide synthesis protocol using the previously (Section 1.2.1) synthesized α-AApeptide building blocks. Each coupling cycle comprised of Fmoc deprotection using 20% piperidine in DMF, and 8 h coupling of 1.5 eq α-AApeptide building blocks onto resin using 4 eq DIC (diisopropylcarbodiimide) / DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. Lipidation was achieved on resin by capping the N-terminus of the final α-AApeptide building block using palmitic acid. The solid support was then transferred into a 4 mL vial and the lipidated α-AApeptides were cleaved from resin by 50:48:2 TFA/CH₂Cl₂ /triisopropylsilane overnight.
Figure 11: Synthetic scheme of NA-75.
1.2.4 Solid phase synthesis of NA-63

NA-63 was synthesized on Rink amide resin in a peptide synthesis vessel, on a Burrell wrist-action shaker. Alloc/Fmoc building blocks (prepared in Section 1.2.2 and shown in Figure 10) were added to the resin, by 8 h coupling of 1.5 eq building block using 4 eq DIC (diisopropylcarbodiimide) and 4 eq DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. Then, Fmoc protection was removed by 20% piperidine in DMF, and hydrocinnamic acid (2 eq) was coupled to the secondary amine,
using 4 eq DIC (diisopropylcarbodiimide) / DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. Alloc protection was removed using 20mg tetrakis(triphenylphosphine)palladium (0), and 100μL phenylsilane. Two more building blocks added similarly. The third building block was capped using acetic anhydride/pyridine, to acetlyate the N-terminus. The solid support was then transferred to a 4 mL vial and the α-AApeptide was cleaved from the resin by 74:24:2 TFA/CH₂Cl₂ /triisopropylsilane for 2 hours. A synthetic scheme of this synthesis is given in Figure 12, on the previous page.

1.2.5 Purification and characterization

Following cleavage of α-AApeptides NA-63, 75, and 77 from solid support, the solvent was evaporated by rotary evaporation and the residues were analyzed and purified on an analytical (1 mL/min) and preparative (20 mL/min) Waters HPLC system using a 5% to 100% linear gradient of 0.1% TFA in acetonitrile in 0.1% TFA in water over 40 min, followed by 100% 0.1% TFA in acetonitrile over 10 min. HPLC traces were detected at 215 nm (UV). All desired fractions were collected, concentrated, and lyophilized. The molecular weights of the α-AApeptides were obtained on a Bruker AutoFlex MALDI-TOF mass spectrometer using α-cyano-4-hydroxy-cinnamic acid matrix.31

1.2.6 Antimicrobial assays

Antimicrobial assays were carried out by other members of our lab, according to the following methods, as reported in our previously published work31.
"The microbial organisms used were *E. coli* (JM109), *B. subtilis* (BR151), multi-drug resistant *S. epidermidis* (RP62A), *C. albicans* (ATCC 10231), Vancomycin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33592), *K. pneumoniae* (ATCC 13383), multi-drug resistant *P. aeruginosa* ATCC 27853. The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits the growth of bacteria in 24 h.

The highest concentration tested for antimicrobial activity was 50 µg/mL. The antimicrobial activities of the lipidated α-AApeptides were determined in sterile 96-well plates by broth micro-dilution method. Bacterial cells 2, 4, 5 and fungi 2, 4, 6 were grown overnight at 37 °C in 5 mL medium, after which a bacterial suspension (approximately 106 CFU/mL) or fungal suspension *Candida albicans* (ATCC 10231) (approximately 103 CFU/mL) in Luria broth or trypticase soy was prepared. Aliquots of 50 µL bacterial or fungal suspension were added to 50 µL of medium containing the α-AApeptides for a total volume of 100 µL in each well.

The α-AApeptides were prepared in PBS buffer in 2-fold serial dilutions, with the final concentration range of 0.5 to 50 µg/mL. Plates were then incubated at 37 °C for 24 h (for bacteria) or 48 h (for *Candida albicans* (ATCC 10231). The lowest concentration at which complete inhibition of bacterial growth (determined by a lack of turbidity) is observed throughout the incubation time is defined as the minimum inhibitory concentration (MIC). The experiments were carried out independently three times in duplicates."
1.2.7 Hemolysis assay

The hemolysis assay was carried out by other members of my research group, according to the following method, as reported in our previously published paper\textsuperscript{31}:

"Freshly drawn human red blood cells (hRBCs) with additive K\textsubscript{2} EDTA (spray-dried) were washed with PBS buffer several times and centrifuged at 1000 g for 10 min until a clear supernatant was observed. The hRBCs were resuspended in 1X PBS to get a 5% v/v suspension. Two fold serial dilutions of α-AAPeptides dissolved in 1X PBS from 250µg/ml through 1.56µg/ml were added to a sterile 96-well plate to make up a total volume of 50 µL in each well. Then 50 µL of 5%v/v hRBC solution was added to make up a total volume of 100 µL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1X PBS and 0.2% Triton-X-100 respectively\textsuperscript{60}. The plate was then incubated at 37°C for 1hr and centrifuged at 3500rpm for 10 min. The supernatant (30 µL) was diluted with 100 µL of 1X PBS and hemoglobin was detected by measuring the optical density at 360nm by Biotek microtiter plate reader (Type: Synergy HT)." \textsuperscript{31}

1.3 Results and Discussion

1.3.1 Synthesis and characterization

Confirmation of the synthesis of NA-75, 77, and 63 was provided by MALDI-TOF, shown in Table 1, below.

Table 1: MALDI-TOF mass spectrometry analysis of α-AAPeptides

<table>
<thead>
<tr>
<th>α-AAPeptide</th>
<th>Formula</th>
<th>Mass calculated</th>
<th>Mass found</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-63</td>
<td>C\textsubscript{62}H\textsubscript{71}N\textsubscript{6}O\textsubscript{7}</td>
<td>1025.54</td>
<td>1049.422 (M+Na)\textsuperscript{+}</td>
</tr>
<tr>
<td>NA-75</td>
<td>C\textsubscript{44}H\textsubscript{87}N\textsubscript{7}O\textsubscript{5}</td>
<td>793.68</td>
<td>795.851 (M+H)\textsuperscript{+}</td>
</tr>
<tr>
<td>NA-77</td>
<td>C\textsubscript{50}H\textsubscript{83}N\textsubscript{7}O\textsubscript{5}</td>
<td>861.65</td>
<td>863.739 (M+H)\textsuperscript{+}</td>
</tr>
</tbody>
</table>
NA-75 and NA-77 were synthesized by the standard method of α-AApeptide synthesis previously reported by our laboratory \(^\text{31}\), with the additional conjugation to a 16-carbon, saturated fatty acid (palmitic acid) on the amino terminus prior to cleavage from the resin. Synthesis of the desired compounds was confirmed by MALDI-TOF MS (mass spectrometry), shown in Table 1.
NA-63 was made by the alternative, alloc/Fmoc α-AApeptide building block method, described in detail in Section 1.2.4. During solid phase synthesis, after each building unit was added to the chain, the Fmoc-protected backbone nitrogen was de-protected and acylated with hydrocinnamic acid. This method was successful in producing the tri-block sequence NA-63, as confirmed by MALDI-TOF MS, shown in Table 1. NA-63, a tri-block sequence is analogous to a hexapeptide, since it has six side-chains on the backbone. Although no further investigation of NA-63 was done, the successful synthesis serves as a proof-of-concept of this synthetic method. It is important to point out that by using this method, a much greater variety of AApeptide sequences can be made with fewer building blocks needing to be prepared first, since half of the side-chains may be selected during the solid phase synthesis. This method may be a viable strategy to improve the efficiency of synthesis in the future – currently, γ-AApeptides are being made by a very similar method. Another enhancement made possible by this method is the combining of Fmoc-deprotection and acylation steps of more than one adjacent building unit, to add the same R-acyl group to each unit. This can reduce the overall time required for synthesis, when adjacent R-acyl groups are repeated.

1.3.2 Antimicrobial and hemolytic activity

NA-75 and 77 showed potent activity against Gram-positive bacteria, with very low hemolytic activity. Activity against Gram-negative bacteria, however, was much lower. Also shown (Figure 14) is the structure and performance of some other lipo-α-AApeptides made by other members of my laboratory, for comparison. Longer, but otherwise similar sequences, such as NB-119-1 and NB-119-2, show higher activity.
Table 2: Antimicrobial and Hemolytic Activity.\textsuperscript{31}

<table>
<thead>
<tr>
<th>Organism</th>
<th>NA-75</th>
<th>NA-77</th>
<th>NB-119-1</th>
<th>NB-127</th>
<th>NB-119-2</th>
<th>NB-119-3</th>
<th>NB-123</th>
<th>Pexiganan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>S. epidermis (MRSE)</td>
<td>10</td>
<td>15</td>
<td>8</td>
<td>20</td>
<td>10</td>
<td>4</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>E. faecalis (VREF)</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>S. aureus (MRSA)</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>8</td>
<td>&gt;50</td>
<td>4</td>
<td>30</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>8</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>20</td>
<td>&gt;50</td>
<td>12</td>
<td>8</td>
<td>10</td>
<td>8-16</td>
</tr>
<tr>
<td><strong>Fungus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>&gt;50</td>
<td>4</td>
<td>20</td>
<td>10</td>
<td>124</td>
</tr>
<tr>
<td>Hemolysis (H$\text{H}_1$/H$\text{H}_0$)</td>
<td>&gt;500/&gt;500</td>
<td>&gt;500/&gt;500</td>
<td>50/300</td>
<td>15/150</td>
<td>20/250</td>
<td>40/400</td>
<td>100/&gt;500</td>
<td>181/495</td>
</tr>
</tbody>
</table>

against Gram-negative bacteria, while retaining activity against Gram-positive bacteria. It is interesting that NB-119-1 is only hydrophobic/cationic building block longer than NA-

Despite the reduced bactericidal activity against Gram-negative bacteria, NA-75 and 77, performed especially well against Gram-positive bacteria and C. albicans, compared not only to the other lipo-AApeptides, but also to the previously reported unlipidated AApeptides. Although the benefits of highly broad-spectrum activity are obvious, there may also be advantages to improving the selectivity between different types of bacteria as well. Therefore the finding that shorter, lipidated AMP mimics have improved activity against Gram-positive bacteria, and reduced activity against Gram-negative may be useful for guiding the tuning of future AMP mimic development for optimal activity against different pathogens.

NA-75 and 77, as well as the longer lipo-α-AApeptides, are expected to make a more extended structure in aqueous solution, rather than a helical structure typical of AMPs, with more conformational flexibility, which may actually assist with bacterial cell
Figure 14: Structures of assayed compounds.\textsuperscript{31}
membrane penetration. Helical structure has been shown not to be necessary for activity, and might cause higher hemolytic activity.\textsuperscript{40,53,54} Some AMPs, such as indolicidin, adopt extended/linear structures rather than helical configurations.\textsuperscript{47} This conformational flexibility may be one of the reasons behind the potent activity of NA-75 and NA-77, and the other lipo-AApeptides.\textsuperscript{31}

Also, in addition to hydrophobic residues on the AApeptide backbone, a lipid tail was introduced on the N-terminus of the AApeptide oligomer. The lipid tail may contribute to the ability of such peptides to interact with the bacterial membrane. The hydrophobic, aliphatic characteristics of the lipid tail may be very beneficial to the proposed membrane disruption mechanism. Compared to previously reported unlipidated antimicrobial AApeptides,\textsuperscript{5,30,31,41} NA-75 and 77 performed especially well against Gram-positive bacteria, and \textit{C. albicans}. In previous work\textsuperscript{5,41}, the shorter 1-3-block sequences, with similar side-chains to NA-77, had very poor antimicrobial activity, with activity drastically increased by increasing the length to 5 or 6 blocks. NA-77 is only a 2-block (tetrapeptide mimetic) sequence, but the addition of a lipid tail results in drastically improved activity (>5-fold), at least against Gram-positive bacteria.\textsuperscript{5,30,31,41}

The potent activity of NA-75 and 77 and the other lipo-\(\alpha\)-AApeptides against \textit{C. albicans} is an indication of the breadth of spectrum that can be obtained by AMP-inspired antimicrobials. Activity against \textit{C. albicans} is significant, because it is a common fungal pathogen. This demonstrates the broad range of microbes AMP mimetics can have activity against.

It is important to note that NA-75 and 77 had quite good activity against strains of \textit{S. aureus}, \textit{S. epidermidis}, and \textit{E. faecalis}, which are multi-drug resistant. MRSA,
MRSE, and VREF, respectively, are each resistant to multiple conventional antibiotics, making infections difficult to treat. As these pathogens become increasingly common, there is an urgent need for the development of antimicrobials which are able to act by means which are unlikely to cause the development of resistance. This is needed both to treat infections by current drug-resistant bacteria and to prevent the development of resistance by new microbes.

Compared to the positive control, pexiganan, the lipo-α-AApeptides performed very competitively. This is significant because it demonstrates that the lipo-α-AApeptide AMP mimetics can have activity competitive with, or better than a current drug candidate. This is an indication that further tuning of the AApeptides could yield clinically useful therapeutic antimicrobials.

Despite the high antimicrobial activity of NA-75 and 77, they have very low hemolytic activity. This selectivity is likely due to interactions between the anionic bacterial membranes, and the basic amino groups of the side-chains, which, in aqueous solution, are protonated, and, thus, cationic. It is thought that this attraction is what affords AMPs their activity and selectivity, and the charge-charge interactions between cationic residues and the membrane is a key initial step, preceding hydrophobic interactions which ultimately damage the membranes, leading to microbial death.

Mammalian cell membranes, which are typically zwitterionic, would not have these attractive charge-charge interactions, and would not be as susceptible to this mode of membrane damage.

Evidence supporting a membrane-disruption mechanism of microbial cell death by lipo-α-AApeptides is provided in our published work, in which a representative lipo-
α-AApeptide, NB-119-2, which had potent activity against both Gram-positive and Gram-negative bacteria, was studied by fluorescence microscopy. It was found to result in increased uptake of propidium iodide dye, after treatment of *E. coli* and *B. subtilis* with NB-119-2, indicating significant damage to the membrane. The aggregation of dead or injured cells was also observed, indicative of a loss of membrane potential. Although fluorescence microscopy studies were not undertaken for NA-75 and NA-77, it is likely that they act by a similar mechanism of action.

### 1.4 Conclusion and Future Work

In conclusion, two lipo-α-AApeptides were identified as having potent antimicrobial activity, especially to Gram-positive bacteria, with very powerful activity against three strains resistant to conventional antibiotics – MRSE, VREF, and MRSA.

Others in my laboratory have concurrently synthesized other AApeptide AMP mimetics, including alpha, gamma, cyclic, and lipidated variations of AApeptides. Many of these have good antimicrobial activity, comparable to pexiganan, currently a drug candidate, with low propensity to cause drug resistance to develop, high selectivity, and stability to proteolytic degradation. AApeptides may provide valuable insights into the development of future antibiotics. There is a great, and urgent need for new antibiotics effective against resistant, virulent bacterial strains. This need is expected to become more severe in the future.

Synthesis of NA-63 from alloc/Fmoc building blocks demonstrates a novel synthetic method for making α-AApeptides. This method may provide greater variety and tunability from a relatively small number of building blocks compared to previously
established methods. It is envisioned that interesting peptides may serve as drug leads for the development of AApeptide analogues, but further study of their secondary structure characteristics is still required in order to improve the predictability and enhance more specific interactions. Synthesis of large libraries of AApeptides for further study is also needed in order to fully realize the potential of these non-natural peptidomimetic oligomers.

This and other work has shown that making peptidomimetic analogues of biologically interesting or potentially useful peptides is one strategy toward modifying their structures in order to make them better potential therapeutics. AApeptides have been developed with potent antimicrobial activity and selectivity while being resistant to proteolytic degradation, and by a mechanism of action which is less likely to lead to antimicrobial resistance. In this and other work, we have shown that rationally designed, bio-inspired AApeptides can have interesting, potent, biomimetic activity. More research needs to be done to enhance the understanding of AApeptide structure and function, in order to further the development of AApeptide-based chemical probes, potential therapeutics, and other bio-interesting compounds.
Chapter 2:
Design and Synthesis of AApeptides for Biomineralization and Self-Assembly to Form Nanostructures

2.1 Introduction

2.1.1 Overview

Molecular self-assembly is ubiquitous and vitally important to natural biological systems. Monomeric units are able to self-assemble through non-covalent interactions to form complex systems, capable of countless unique biological functions. Hierarchical assembly occurring in nature is exhibited by such vitally important biological components as lipids, proteins, and nucleic acids. Understanding molecular self-assembly is very important to the construction of novel or useful nanostructures and nanomaterials. Peptide-based nanomaterials are currently being explored for applications to nanotechnology, nanomedicine, etc. In aqueous solutions, peptide amphiphiles have been used to generate self-assembled nanostructures, forming a variety of nanostructures, including nanotubes, nanorods, nanovesicles, micelles, nanobelts, and nanofibers. Non-natural amphiphilic peptidomimetic oligomers may potentially lead to new biomimetic nanomaterials with novel functionality. Here, we report the synthesis of novel amphiphilic peptidomimetics based upon our recently developed α- and γ-AApeptide scaffolds and their ability to form nanostructures by molecular self-assembly. As a test of their possible biomimetic applicability, we studied
the effect of these compounds on the mineralization of CaCO$_3$ in aqueous solution.

Biomineralization of CaCO$_3$ is an important biological process, and research into CaCO$_3$ mineralization may provide insights into CO$_2$ storage, one possible strategy for combating global warming.$^{42}$

![Figure 15: α-Peptides, α-AApeptides, and γ-AApeptides.](image)

Herein we report the novel synthesis of several AApeptides, and subsequent investigation of their potential utility. AApeptides are a new class of peptide mimics developed by our laboratory, derived from the alpha-chiral PNA (peptide-nucleic acid) backbone, shown in Figure 4.$^3$ AApeptides are oligomers of N-acylated-N-aminoethyl amino acids, first reported by members of my research group in 2011.$^3,4$ Our laboratory reported the first synthesis of both α-AApeptides and γ-AApeptides, which are very
similar, but differ in the positioning of side-chains along the backbone. α-AA peptides are discussed in greater detail in Chapter 1 of this manuscript. γ-AA peptides differ from α-AA peptides in that the amino acid-derived side chain is at the γ position of each building block rather than the α-position. As a result, γ-AA peptides are expected to have greater conformational freedom.4

2.1.2 Self-assembly of peptide amphiphiles

Molecular self-assembly is a natural process which vitally important to, and universal among organisms of all types. Noncovalent interactions between molecules allow them to self-assemble, forming complex systems from simple monomeric subunits.65 A very relevant example of such self-assembled structures in nature is the lipid membrane, which encloses every cell and is a structure vital to all organisms. This type of hierarchical self-assembly is also exhibited by proteins and nucleic acids.66 Understanding the mechanisms of biomolecular self-assemble is important for the advancement of nanotechnology research, such as the development of novel nanostructures and nanomaterials.67 By elucidating mechanisms of molecular self-assembly, new monomeric subunits may be developed, yielding novel nanomaterials with new and interesting functionality. Peptide amphiphiles have been of interest in bioorganic and chemical biology, and have been used to form nanostructures in aqueous solution. Typically, peptide amphiphiles are composed of a hydrophilic "head" region, and a hydrophobic "tail" region.75 The hydrophilic region typically consists of charged or polar amino acids, while the hydrophobic region may contain alkyl chains, lipids, or hydrophobic amino acids. Peptide amphiphiles have been shown to form a wide array of
nanostructures, including membranes, micelles, vesicles, nanorods, nanofibers, nanotubes, and nanobelts.  

Biocompatible nanostructures formed by self-assembly of short peptide amphiphiles may find utility in drug delivery or regenerative medicine. Some peptide amphiphiles have been used as scaffolds for biomineralization of hydroxyapatite, which may further research into enamel and bone regeneration. In addition to influencing biomineralization, peptide-based nanostructures may be useful for drug delivery. One approach to drug delivery is the sequestration of drug molecules in liposomes or similar amphiphilic nanoparticles, which would then release the drug molecules some time after administration. Of especially great interest in pharmacology is targeted/triggered drug delivery, in which drug molecules are only released at a target area of the body. This may be accomplished in self-assembled nanostructures by designing monomers which disassemble under certain conditions. Such nanostructures would retain the drug molecules until encountering some stimulus which causes the structure to disassemble, releasing the drug molecules into solution. Hydrophobic drug molecules may be especially amenable to this, since they could simply be retained in the hydrophobic inner region of the nanostructure.

pH plays an important role in the self-assembly of peptide amphiphiles in aqueous environment. The hydrophilic region of peptide amphiphiles often contains basic or acidic residues such as lysine or arginine (basic) or aspartic or glutamic acid (acidic). Due to these basic or acidic groups, self-assembly may be affected by pH. For example, a peptide amphiphile reported by Stupp, et al, containing a number of acid groups and a lipid tail, was able to self-assemble only under low pH (<4). Under neutral
(physiological) pH, the peptide amphiphile would not be stable, and, therefore, ineffective as a means of drug delivery. Peptide-based amphiphiles which self-assemble at physiological pH but disassemble at high or low pH, however, may be capable of drug delivery triggered by pH.\textsuperscript{77}

### 2.1.3 Biomineralization using peptides/peptidomimetics

Biomineralization is an important biological process occurring in a wide variety of organisms, in which peptides and proteins play a very significant role. Biomineralization is the process by which organisms produce minerals, for a variety of functions. The most common types of biominerals are calcium salts, especially those of phosphate and carbonate.\textsuperscript{78-81} Organisms are able to produce complex structures from the nanometer to macroscopic level, with precise control of morphology.\textsuperscript{80,81} Peptides play an important role in the formation of structures made from the biomineralization of calcite, calcium phosphates, silicates, and other biominerals. Biominerals are often used by organisms as skeletons, for structural support, such as hydroxyapatite in bones and teeth, or silica of the cell walls of diatoms.\textsuperscript{80,81} The long-extinct trilobites even possessed complex, compound eyes with lenses made of transparent calcite.\textsuperscript{82} Other organisms, including some bacteria and animals, produce magnetite, an iron mineral which allows magnetoception, the ability to sense magnetic fields.\textsuperscript{83}

Materials or compounds capable of controlling, directing, or catalyzing mineralization could have many potential uses. One very important potential use is the mineralization of CaCO\textsubscript{3}, especially from atmospheric/gaseous CO\textsubscript{2}, since it is a "greenhouse" gas, and global warming is an increasingly significant issue. CO\textsubscript{2}
capture/storage strategies are currently being explored for combating global warming. One way of potentially reducing the amount of CO$_2$ in the atmosphere might be by first hydrating it to convert it to carbonic acid, then forming a solid salt or mineral, which could then be stored indefinitely. Catalysts of mineral formation from CO$_3^{2-}$ and various cations (Ca$^{2+}$ in the case of CaCO$_3$) in aqueous solution are desirable, to give control over the rate of crystallization, as well as influence over the morphology, size, etc.$^{80,81}$ Fine control over mineral morphology may be interesting for nanomaterials research. CaCO$_3$ is also an interesting biomineral in its own right, forming shells of marine organisms, and many other important biological structures.$^{84}$

Hydroxyapatite is an especially important biomineral to humans, since it is what teeth and bones are primarily composed of ($>50\%$).$^{85}$ Stupp, et al used peptide amphiphile nanofibers to show that hydroxyapatite mineralization can take place on a self-assembled nanofiber displaying anionic carboxylate and phosphate groups. They proposed that mineralization may be induced by local ion supersaturation near the surface of the nanofibers, facilitated by the presence of carboxylate and phosphate groups in the hydrophilic region of the peptide amphiphiles.$^{76}$ They found that the crystallographic $c$-axes of the hydroxyapatite crystals were oriented aligned with the long axes of the nanofibers, indicating that some kind of templating occurred during mineralization. This type of biomimetic control of hydroxyapatite mineralization on non-natural amphiphilic nanostructures is interesting in biomedical research into bone or enamel regeneration.$^{75}$

Although both peptides and peptidomimetics have been studied as potentially promoting bone mineral formation by promoting activity of osteoblasts,$^{86}$ the study of the direct promotion of mineralization by peptidomimetics is rarely reported.$^{87}$ Amphiphilic
peptoids have been developed by Chen, et al, which are able to dramatically affect mineralization of calcium carbonate, both by altering the morphology of the calcite crystals, as well as by accelerating crystal growth, even at very low (<50nM) concentrations. This is despite the lack of any apparent formation of self-assembled structures in solution. The group proposed several mechanism of accelerated crystal growth. The first is essentially the same mechanism proposed by Stupp, et al – supersaturation by increasing the local concentration of Ca\(^{2+}\) ions near the peptoids. The other mechanisms suggested involve reducing the activation barrier for crystal formation, either through interactions between the peptoids and ions, or through disruption of interaction of water on the crystal surface.

Much of the investigation of oligomeric amphiphiles for biomineralization is so far focused on L\(\alpha\)-peptides or their immediate derivatives, such as the amphiphilic peptides and lipopeptides. As discussed previously in Chapter 1, natural \(\alpha\)-peptides are often susceptible to proteolytic degradation and may be immunogenic. Therefore, the development of new peptidomimetics that may have similar ability to influence mineral growth and morphology is needed.

**2.1.4 Design and synthetic strategy**

**2.1.4a NA-75, 77, and 81**

Although the design of AApeptides for AMP mimetic and self-assembly/mineralization efforts are guided by different processes and properties, amphiphilicity appears to be a key factor for both. NA-75 and 77 were initially synthesized for study as antimicrobials, as described in Chapter 1, and are short,
lipidated, amphiphilic, α-AApeptides. Even though NA-75 and 77 were designed as AMP mimetics, they also possess many of the characteristics of compounds which may self-assemble in aqueous solution or promote biomineralization, so they were also studied in this project. NA-81 (shown in Figure 19) was also synthesized, according to the same methodology as NA-75/77. NA-81 and NA-75 and 77 are very similar in overall structure, with the main difference being that NA-81 is anionic, while NA-75 and 77 are cationic. The different properties of these compounds may give insight into the importance of AApeptide charge on their self-assembly and mineralization behavior.

It was reasoned that these amphiphilic AApeptides with lipid "tails" and hydrophilic "heads" might self-assemble in aqueous solution, driven by the alkyl chains packing together into the center of micelles or other nanostructures, and the anionic or cationic groups will remain exposed to the water solution.75,76 These initially formed nanostructures may then be able to aggregate further to form larger, more complex nanostructures.

TEM was used to observe any nanostructures formed by self-assembly of the amphiphilic oligomers. Optical microscopy was used to observe CaCO₃ crystals formed in the presence of the AApeptide amphiphiles.

2.1.4b NA-133, 135, 137, and 139

Four new amphiphilic γ-AApeptides were designed and synthesized in order to investigate their self-assembly in aqueous media and ability to influence CaCO₃ mineralization. The structures of these compounds are given in Figure 16. The new γ-
<table>
<thead>
<tr>
<th>Name</th>
<th>Arrangement of charged groups</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-133</td>
<td>++ + - - -</td>
<td><img src="image1.png" alt="Structure" /></td>
</tr>
<tr>
<td>NA-135</td>
<td>- - - + + -</td>
<td><img src="image2.png" alt="Structure" /></td>
</tr>
<tr>
<td>NA-137</td>
<td>+ + + + + + +</td>
<td><img src="image3.png" alt="Structure" /></td>
</tr>
<tr>
<td>NA-139</td>
<td>- - - - - -</td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
</tbody>
</table>

**Figure 16: NA-133, 135, 137, and 139.**

AApeptides were made by the standard building block method, from 2 different γ-AApeptide building blocks: one derived from phenylalanine/γ-aminobutyric acid, and one from phenylalanine/succinic acid. Two of the sequences, NA-137 and 139, were each made from a single repeated building block. NA-133 and 135, however, were made from an alternating pattern of the two building blocks, as shown below. NA-137 and 139 may undergo self-assembly in aqueous solution due to hydrophobic/hydrophilic interactions.
with water. The non-polar phenyl side-chains may aggregate in the center of nanostructures, while the anionic or cationic side-chains are expected to remain exposed to the aqueous environment. NA-133 and 135 are expected to behave similarly, but with additional intermolecular interactions made possible by the alternating cationic/anionic side-chains. This may allow the formation of nanostructures with zwitterionic surface charge, rather than overall cationic or anionic, which may have some desirable effects, especially with regard to the pH at which self-assembly takes place.

TEM was used to observe any nanostructures formed by self-assembly of the amphiphilic oligomers. Optical microscopy was used to observe CaCO₃ crystals formed in aqueous solution in the presence of the AApeptide amphiphiles.

2.1.4c NA-137+139

Additionally, a 1:1 mixture of NA-137 (all positively charged side-chains) and NA-139 (all negatively charged side-chains) was prepared, since we believed the complementary charges of the amino and carboxylate groups might lead to interesting interactions, giving rise to unique self-assembly or mineralization behavior. Together, they might show an increased propensity to self-assemble, due to electrostatic interaction between oppositely charged side chain groups. The 1:1 mixture (referred to herein as NA-137+139) was studied by TEM to observe any nanostructures formed. Optical microscopy was used to observe CaCO₃ crystals formed in aqueous solution in the presence of the mixture.
2.2 Experimental

2.2.1 \(\gamma\)-AApeptide building block synthesis

Reductive amination of glycine benzyl ester hydrochloride (neutralized with TEA) was carried out using 1 eq Fmoc-phenylalanine aldehyde, \(^{65,66}\) 2 eq NaCNBH\(_3\) and a catalytic amount of acetic acid.\(^{64,65}\) Following purification by flash chromatography using 1:1 hexane:ethyl acetate, the resulting secondary amine was then acid coupled to succinic acid mono-\(r\)-butyl ester (2 eq) using 2 eq DhbtOH (3-hydroxy-1,2,3-benzotriazin-4(3H)-one) and 2 eq DIC (N, N’-Diisopropylcarbodiimide) as activation agents. The final step was hydrogenation, which was carried out in the presence of H\(_2\) gas and 20% palladium on activated carbon as catalyst in methanol. The other building block, shown in Figure 17 was prepared similarly, but by acylating the secondary amine with Boc-protected \(\gamma\)-aminobutyric acid instead.

![Figure 17: \(\gamma\)-AApeptide building blocks.](image)

2.2.2 Solid phase synthesis of NA-133, 135, 137, and 139

\(\gamma\)-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels on a Burrell wrist-action shaker following the standard Fmoc chemistry solid phase peptide
Figure 18: Synthetic scheme of NA-133.
synthesis protocol using the previously (Section 2.2.1) synthesized γ-AApeptide building blocks (shown in Figure 17). 3,4,42,47-49

Each coupling cycle comprised of Fmoc deprotection using 20% piperidine in DMF, and 8 h coupling of 1.5 eq γ-AApeptide building blocks onto resin using 4 eq DIC (diisopropylcarbodiimide)/DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. The solid support was then transferred to a 4 mL vial and the γ-AApeptides were cleaved from resin by 74:24:2 TFA/CH₂Cl₂/triisopropylsilane for 2 hours. 4,88

2.2.3 Solid phase synthesis of NA-75, 77, and 81

Lipidated α-AApeptides were prepared on Rink amide resin in peptide synthesis vessels on a Burrell wrist-action shaker following the standard Fmoc chemistry solid phase peptide synthesis protocol using the previously (Section 1.2.1) synthesized α-AApeptide building blocks. 3,4,42,47-49

Each coupling cycle consisted of Fmoc deprotection using 20% piperidine in DMF, followed by 8 h coupling of 1.5 eq α-AApeptide building blocks onto the resin using 4 eq DIC (diisopropylcarbodiimide) and 4 eq DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. Lipidation was achieved on resin by capping the N-terminus of the final α-AApeptide building block using palmitic acid (C₁₆). The dried solid support was then transferred into a 4 mL vial and the lipidated α-AApeptides were cleaved from the resin by 50:48:2 solution of TFA/CH₂Cl₂/triisopropylsilane overnight. 3,4,31,42,47-49
2.2.4 Purification and characterization α- and γ-AApeptides

Following cleavage of AApeptides from solid support, the solvent was evaporated by rotary evaporation and the residues were analyzed and purified on an analytical (1 mL/min) and preparative (20 mL/min) Waters HPLC system using a 5% to 100% linear gradient of 0.1% TFA in acetonitrile in 0.1% TFA in water over 40 min, followed by 100% 0.1% TFA in acetonitrile over 10 min. HPLC traces were detected at 215 nm (UV). All desired fractions were collected, concentrated, and lyophilized. The molecular weights of the AApeptides were obtained on a Bruker AutoFlex MALDI-TOF mass spectrometer using α-cyano-4-hydroxy-cinnamic acid matrix.³,⁴,³₀,³¹

2.2.5 TEM studies

Samples were applied to TEM grids by adding 10μL AApeptide solution, and the grids were allowed to dry for about 1h. After drying, the grids were stained with 10μL of 1% (w/w) uranyl acetate/water solution, and extra solution was removed by immersion using wetfilter paper after 30s. The grids were allowed to dry prior to TEM study. TEM images were obtained on a FEI Morgagni 268D TEM with an Olympus MegaView III camera on the microscope. The microscope uses AnalySiS software to run the camera and was operated at 60 kV.⁴²

2.2.6 CaCO₃ mineralization studies

(NH₄)₂CO₃ vapor was diffused over a 96-well plate, in which other wells contained 198μL of 5.0 mM CaCl₂ solution and 2μL of 1 mg/mL AApeptide, giving a final concentration of 10μg/mL. The control did not contain AApeptide; instead, 2 μL of
water was added. The 96-well plate was placed in a closed Ziploc bag for ~ 24 hours to allow \((\text{NH}_4)_2\text{CO}_3\) vapor diffusion. The morphology of crystals was investigated by optical microscopy.42,87

2.3 Results and Discussion

2.3.1 Synthesis and characterization

Confirmation of the synthesis of the AApeptides was provided by MALDI-TOF, found in Table 3, below.

Table 3: MALDI-TOF mass spectrometry analysis of AApeptides.

<table>
<thead>
<tr>
<th>AApeptide</th>
<th>Formula</th>
<th>Mass calculated</th>
<th>Mass found</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-75</td>
<td>C_{44}H_{87}N_{7}O_{5}</td>
<td>793.68</td>
<td>795.851 (M+H)^+</td>
</tr>
<tr>
<td>NA-77</td>
<td>C_{50}H_{83}N_{7}O_{5}</td>
<td>861.65</td>
<td>863.739 (M+H)^+</td>
</tr>
<tr>
<td>NA-81</td>
<td>C_{33}H_{69}N_{5}O_{9}</td>
<td>739.51</td>
<td>763.646 (M+Na)^+</td>
</tr>
<tr>
<td>NA-133</td>
<td>C_{90}H_{120}N_{16}O_{18}</td>
<td>1712.90</td>
<td>1714.071 (M+H)</td>
</tr>
<tr>
<td>NA-135</td>
<td>C_{90}H_{120}N_{16}O_{18}</td>
<td>1712.90</td>
<td>1714.089 (M+H)</td>
</tr>
<tr>
<td>NA-137</td>
<td>C_{90}H_{129}N_{19}O_{12}</td>
<td>1668.01</td>
<td>1670.099 (M+H)</td>
</tr>
<tr>
<td>NA-139</td>
<td>C_{90}H_{111}N_{15}O_{24}</td>
<td>1757.79</td>
<td>1780.882 (M+Na)^+</td>
</tr>
</tbody>
</table>

NA-75, 77, and 81 were synthesized by the standard method of \(\alpha\)-AApeptide synthesis previously reported by our laboratory,³ and discussed in Chapter 1, with the additional conjugation to a 16-carbon, saturated fatty acid (palmitic acid) on the amino terminus prior to cleavage from the resin.³¹ NA-133, 135, 137, and 139 were prepared by the standard \(\gamma\)-AApeptide synthesis previously reported by our laboratory.⁴ Synthesis of the desired compounds was confirmed by MALDI-TOF MS, shown in Table 3.
<table>
<thead>
<tr>
<th>NA-75</th>
<th><img src="image" alt="Structure" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-77</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>NA-81</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>NA-133</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>NA-135</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>NA-137</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>NA-139</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

Figure 19: Structures of synthesized α-AAs peptides.
2.3.2 TEM studies of self-assembly by AApeptide amphiphiles

Figure 20: TEM of NA-133.

Figure 21: TEM of NA-135.
Figure 21 (Cont.): TEM of NA-135.

Figure 22: TEM of NA-137.

Figure 23: TEM of NA-137+139.
TEM of the AApeptide amphiphiles are shown in the figures above. No nanostructures formed from NA-75, 77, 81, or NA-139 were observed by TEM, but the γ-AApeptide amphiphiles NA-133, 135, 137, and the mixture NA-137+139 clearly formed some interesting nanostructures. NA-133 and 135 formed small (< 50nm) nanoparticles. The mixture NA-137+139 formed large nanorods. Several of the TEM images of NA-137+139 show what may be smaller nanostructures aggregating to form the larger nanorods, hinting at a hierarchical mechanism of self-assembly. The TEM images of NA-137 are not as clearly defined, but they may show small nanoparticles or nanofibers.
It is important to note that these nanostructures were formed at pH 7. Many peptide amphiphiles are generally anionic or cationic and self-assemble only at high or low pH. Purely anionic amphiphiles often only form stable nanostructures under low pH\textsuperscript{76}, but disassemble at neutral (physiological), or basic pH. The nanostructures of the zwitterionic NA-133, 135, and 137+139 are formed at neutral or near-neutral pH. It may be possible to further develop such AApeptide amphiphiles tuned to form stable zwitterionic nanostructures at neutral pH, but break down into their subunits under acidic or basic conditions. Nanostructures which can be tuned to disassemble under certain conditions are of great interest to chemical biology, because they may be useful for stimulus-triggered drug delivery, if drug molecules can be introduced into the hydrophobic inner region of the nanostructure.\textsuperscript{67}

The hydrophobicity imparted by the phenylalanine-derived side-chains appears to be capable of sufficiently driving aggregation of the AApeptides in aqueous conditions. Charge-charge interactions between the zwitterionic \(\gamma\)-AApeptides may also help to stabilize the aggregation (at least at neutral pH).

This is supported by our observation that the zwitterionic \(\gamma\)-AApeptides NA-133 and 135, as well as the complementarily-charged mixture of NA-137+139, have a higher propensity to form defined nanostructures than the fully anionic or cationic counterparts alone.

### 2.3.3 Calcium carbonate mineralization

Calcium carbonate mineralization was done by diffusion of CO\textsubscript{2} vapor from ammonium carbonate, into aqueous solution containing CaCl\textsubscript{2} and AApeptide (control
had water instead of AApeptide.) There were no subjective differences in the overall number or size of the CaCO₃ crystals detectable by visual microscopy between the AApeptides and each other or the control (H₂O solution). The rhombohedral crystals typical of calcite are visible in the control sample. CaCO₃ crystals grown in the presence of many of the AApeptides appear to aggregate together, forming clusters of CaCO₃ crystals. The morphology of the CaCO₃ crystals grown in the presence of AApeptides

![Figure 24: CaCO₃ mineralization control.](image)

![Figure 25: CaCO₃ mineralization of NA-75.](image)
Figure 25 (Cont.): CaCO₃ mineralization of NA-75.

Figure 26: CaCO₃ mineralization of NA-77.
Figure 27: CaCO$_3$ mineralization of NA-81.

Figure 28: CaCO$_3$ mineralization of NA-133.
Figure 29: CaCO$_3$ mineralization of NA-135.

Figure 30: CaCO$_3$ mineralization of NA-137.
Figure 31: CaCO₃ mineralization of NA-139.

Figure 32: CaCO₃ mineralization of NA-137+139.
were markedly different from the rhombohedral crystals of the control. NA-75 resulted in spherical CaCO$_3$ crystals, which aggregate together. NA-77 and several others resulted in irregularly shaped structures. Clearly, the AApeptide amphiphiles are able to have a significant impact on the morphology of the calcium carbonate crystals.

Nanostructures formed from AApeptides which are negatively charged at pH 7 would be expected to induce CaCO$_3$ mineralization by similar mechanisms to anionic peptide amphiphiles – nucleating crystal formation by local supersaturation of ions. Cationic or zwitterionic AApeptides also influence CaCO$_3$ mineralization, perhaps through a similar ion supersaturation mechanism. While anionic sequences may cause supersaturation of Ca$^{2+}$ ions, cationic sequences could similarly cause supersaturation of their counter-ion, leading to mineral nucleation. Zwitterionic nanostructures, such as those formed by NA-137+139, present another interesting possibility – a mechanism by which both cations and anions are templated by the anionic and cationic residues at the surface nanostructures. While few of the AApeptide amphiphiles formed nanostructures, nearly all of them had some influence on CaCO$_3$ crystal morphology. It is
therefore impossible to draw any firm conclusions about the effects of nanostructure morphology on CaCO\textsubscript{3} mineralization without further study.

2.4 Conclusion and Future Work

We have demonstrated the ability of rationally designed, bio-inspired, novel AApeptide amphiphiles to form nanostructures by molecular self-assembly. Several γ-AApeptides, NA-133, 135, and a 1:1 ratio of NA-137 and 139 were observed to form nanostructures in aqueous solution. NA-137+139 formed large nanorods, and NA-133 and 135 formed spherical nanoparticles, possibly micelles or liposomes.

Furthermore, we have demonstrated the ability of these and other AApeptide amphiphiles to influence CaCO\textsubscript{3} mineralization. CaCO\textsubscript{3} crystals formed in the presence of a number of AApeptide amphiphiles were found to have different morphology than typical rhombohedral calcite crystals. This finding is significant to the development of novel peptide-based nanomaterials to undergo mineralization of biologically relevant minerals. The ability for the AApeptides to influence CaCO\textsubscript{3} mineralization may also be relevant to research into CO\textsubscript{2} capture and storage, since the source of carbonate for the mineralization was gaseous CO\textsubscript{2}, diffused over the aqueous solution. This is an important area of research, because sequestration of atmospheric CO\textsubscript{2} is one strategy to alleviate global warming.\textsuperscript{87}

The properties of AApeptide amphiphiles and their nanostructures still require further study. Study of the nanostructures to investigate their possible utility for triggered drug delivery would also be interesting, since there is a need for tunable, biodegradable, vehicles for drug delivery.\textsuperscript{87} Kinetic study of CaCO\textsubscript{3} mineralization in the presence of our
AApeptide amphiphiles to determine whether the rate of CaCO₃ mineralization can be improved by such systems is also needed.⁸⁷ Also of interest is hydroxyapatite biomineralization, which may be important to research into bone or enamel regeneration.⁷⁵,⁷⁶
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