Harnessing the Power of Fluorination for Protein Engineering

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Harnessing The Power Of Fluorination For Protein Engineering

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May, 2009
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I. ABSTRACT

A common method of studying proteins is to introduce mutations into the amino acid sequence of the system. Incorporating phenylalanine analogs of varying degrees and sites of fluorination on the aromatic system gave substantial insight into the structure—function relationship of model peptide systems. By strategically placing tetrafluorinated phenylalanine mutants into the villin headpiece, HP35, increased thermodynamic and thermal stability was achieved. Using these *highly but not fully* fluorinated novel amino acid analogs allowed for the retention of the important ArH···π interactions of the system. Furthermore, fluorinated amino acid residues were introduced into peptide systems known to form pores in lipid membrane systems. Certain fluorinated mutants of the membrane pore-forming peptides (MPP) showed increased membrane activity. Thus, fluorinated amino acids have tremendous potential to create hyperstable protein conformations, as well as increase the activity of proteins in membranes.
2. INTRODUCTION

2.1 Protein—Membrane Interactions

A biological membrane is the barrier between the extracellular environment and the cell itself. It is the membrane that keeps potential toxins out of the cell and essential nutrients within the cell. The membrane is also the site of communication for the cell, hosting both receptors and transmitters, allowing the cell to live in harmony with its surroundings. While the membrane acts as the medium for these functions, it is proteins that are the vehicles for performing these tasks. Proteins may act as receptors, pumps, channels, scaffolds and many other countless duties of the cell when anchored to the membrane. Due to the breadth of relationships between them, there are very specific interactions that occur between proteins and membranes. Not all proteins have the ability to interact with or within a membrane, as the biological membrane is a truly unique region of the cell. There are specific properties of membrane proteins that allow them to have favorable interactions with the biological membrane.

Two large classes of membrane proteins exist: peripheral membrane proteins and integral membrane proteins. Peripheral membrane proteins only temporarily interact with the membrane, transiently associating with outer parts of the membrane. Integral membrane proteins, on the other hand, are permanently attached to the biological membrane with which it interacts. Many integral membrane proteins are also transmembrane proteins, which span the entire length of the lipid bilayer at least once. This group of proteins can be subdivided even further, based upon their conformation of
secondary structure in the membrane, as transmembrane proteins can exist as either α-helical or β-sheet structures.

It is clear that membrane proteins must have certain properties that allow them to effectively partition into the membrane, which is a task many proteins cannot accomplish. The membrane, a lipid bilayer, is stratified into different regions that account for a set of unique environments within a small space. The lipids composing the membrane consist of two parts: a polar head group and a long, hydrophobic chain. The membrane orients itself so that the aliphatic chains cluster together to form an extremely hydrophobic core for the membrane. On the outer portion of the bilayer, the polar head groups join together to create the membrane interface region. As the immediate region where the membrane meets extracellular environment, the interface is the initial site of protein-membrane interaction. Further, the membrane interface region accounts for 50% of the thermal thickness of the bilayer.¹

Interestingly, there are characteristic interactions between proteins and membranes that occur at the membrane interface region. Most notably is the localization of aromatic residues at the membrane interface. A statistical survey of known membrane protein structure and of genomic databases indicates that aromatic residues are not uniformly distributed within membrane proteins, but rather are extensively concentrated at the interfacial region of membranes.

Aromatic amino acids very commonly interact with one another within a protein in order to enhance the overall stability of the protein.² Aromatic-aromatic interactions, driven by van der Waals or electrostatic forces aid to the overall stability of membrane proteins by clustering together in the chemically unique environment of the membrane.³
Further, it has been noted that these aromatic residues act as an “aromatic belt,” or anchor,\textsuperscript{4} keeping the protein lodged in its intended orientation. Another large involvement of the aromatic regions around the membrane interface is the ability to contribute to the hydrogen-bonding network. At the membrane interface, aromatic residues are capable of forming hydrogen-bonding networks with lipid carbonyls or interfacial water molecules.\textsuperscript{5} Additionally, dipole—dipole interactions can aid in the partitioning of proteins into the membrane system.\textsuperscript{3}

Moreover, the hydrophobicity of the residues involved in partitioning into the membrane plays a crucial role into the stability of a membrane protein. This “hydrophobic effect” was experimentally quantified through a series of thermodynamic experiments by Wimley and White.\textsuperscript{6} From these studies, the Wimley-White (WW) interface hydrophobicity scale was determined. The WW scale insists that a hydrophobic residue is more thermodynamically favorable in a membrane interface than a less hydrophobic moiety. Taken together, aromatic interactions and hydrophobicity are critical driving forces in the localization and stability of membrane proteins.

2.2 Antimicrobial Peptides

The highly specific interactions between proteins and membranes have been exploited by many biological systems to create an innate form of defense for organisms across the animal and plant kingdoms. Known as antimicrobial peptides (AMPs), this class of peptides act as toxins against a wide range of invasive microbes. This supplies the host with an effective first line of defense against invasive microbes. Due to the
presence of AMPs, organisms such as plants, without a complex immune system, can live harmoniously among a vast collection of invasive pathogens.

Of the hundreds of characterized AMPs, there has been no observed sequence or structural homology throughout the diverse range of effective AMPs. Instead, it has been postulated that the composition of the short peptides is what is essential for the AMPs to effectively partition into the membrane and carry out its duties as an antimicrobial agent. The peptide must be composed of a stretch of hydrophobic residues to interact with the long, amphipathic center of the membrane. Additionally, the peptides are often extremely rich in aromatic residues, which account for favorable interactions at the membrane-interface region. Due to the lack of sequence and structural homology across this wide range of peptides, it is not feasible to predict the potency of a specific sequence targeted for antimicrobial response.

While the mechanism of AMP toxicity is variable and not completely deciphered, it is understood that upon partitioning into the membrane of a microbe, pores may form. This pore often causes a large enough conformational change in the membrane, allowing the contents of the cell to leak, or even allowing small molecules to enter into the cell. While the cause of necrosis can vary, it is associated with the insertion and pore-formation of the AMP into the cellular membrane. Upon inserting into the membrane, the AMP can cause a considerable amount of strain of the lipid bilayer, transforming the membrane into a permeable structure allowing molecules to pass out from or into the cell.

While no structural homology exists across this class of peptides, both β-sheet- and α-helical-rich pores have been observed to partition and assemble themselves into
the biological membrane. The AMPs are generally water soluble, and the β-strand structures often have the propensity to assemble at the membrane interface region before inserting into the membrane due to their ability to form side-by-side hydrogen bonding networks.

2.3 Amyloid Toxicity

There are several parallels between Alzheimer’s disease (AD) and the function of AMPs, as the formation of toxic pores by peptides into cell membranes has been implicated as a key step in AD pathology. Clinically, AD is a neurodegenerative disease that affects over 26 million people worldwide. This deadly disease is characterized by the progressive deterioration of cognitive function. Currently, there is no cure for AD, and the few available therapeutics are limited to the role of providing brief relief to the symptoms of the disease.

Along with many other diseases, such as Huntington’s disease, Mad Cow Disease and type II diabetes, AD is part of a class of amyloidogenic diseases. An amyloid is a highly ordered proteinaceous mass, or aggregate, which forms fibrils approximately 60 to 130 Å wide. Amyloid fibrils were first discovered by Rudolph Virchow in 1854, who originally mistook the aggregated proteins as carbohydrates. In order to be classified as an amyloid, a protein aggregate must adhere to three general principles: it forms fibers, visible by electron microscopy, it binds to the dye Congo red, and it must be a β-sheet-rich structure.

Further, AD is distinguished by the accumulation of extracellular fibrillar amyloid plaques in the brain. These amyloid plaques are largely composed of polymers of
amyloid-β (Aβ), a 39-43 residue peptide.\textsuperscript{16} Aβ is the product of cleaved amyloid precursor protein (APP), a naturally existing peptide in the brain, which is subject to cleavage by β- and γ-secretases.\textsuperscript{16} Once cleaved, Aβ has the propensity to polymerize into increasingly ordered structures. Aβ oligomerizes first into dimers, then spheres, followed by the formation of chain-like protofibrils and finally into fibrils. The accumulation of these fibers as neurological tangles and plaques is often the hallmark of the onset of AD.

There is strong support however, that the fibrils themselves are not toxic, but the preceding oligomers are the cause of neurotoxicity.\textsuperscript{17, 18} One strong piece of evidence supporting this claim is that there is no correlation between the amount of amyloid fibrils formed in the brain and the severity of the disease.\textsuperscript{19} There is, however, compelling evidence that the amount of soluble Aβ (monomers and protofibrils) is directly correlated to the clinical severity of the disease.\textsuperscript{20}

The refined theory of Lashuel and Lansbury, known as the “channel hypothesis,” elaborates on the toxicity of the protofibrils. This theory suggests that Aβ, in fact, acts analogously to an AMP, but instead of forming pores within the membranes of invasive cells, proves to be toxic to neuronal host cells. The argument is supported by the fact that before the formation of the highly ordered fibrils, β-sheet rich oligomeric Aβ intermediates exist as precursors.\textsuperscript{21} This is akin to the mechanism of many AMPs, which oligomerize into β-sheet-rich structures before inserting into a membrane and compromising the integrity of the cell. The channels formed by Aβ are capable of disrupting the Ca\textsuperscript{2+} homeostasis of cells, which has been a longstanding characteristic of
AD pathogenesis. The disruption of the electrochemical gradient of a neuronal cell will prevent it from performing its function.22

While the channel hypothesis is not universally accepted as the main and sole root of AD pathology, there are many studies that reinforce the hypothesis. Regardless, the possibility of the channel hypothesis alone asserts that it is exceedingly important to gain a better understanding of peptide—membrane interactions, with special attention paid to the formation of pores. By understanding the mechanism of peptide pore-formation, there is immense potential for biomedical applications. On one hand, it is theoretically possible to design an AMP mimic, which is extremely potent and essentially immune to bacterial resistance to act as a super-antibiotic. This is an exceedingly important issue, as bacterial resistance to common antibiotics is becoming a significant public health concern. On the other hand, by understanding the mechanism of peptide pore-formation, it is possible to develop a method to inhibit or block the event of pore-formation. This would be of use for the prevention or cure of amyloidogenic diseases, such as AD, which follow the channel hypothesis.

2.4 Experimental Design: Membrane Pore-Forming Peptides

There are well-characterized methods to study protein folding and stability in solution. Once incorporated into a membrane system, proteins become extensively more difficult to study—there are significantly more variables such as the lipids and buffers, in addition to the aberrant behavior of proteins in this unique environment. Therefore, it is much easier to study the structure-function relationship of well-characterized natural protein systems. However, the aforementioned benefits of studying membrane pore-
forming peptides (MPPs) insist that de novo design of MPPs is of interest, since they can be exploited to act as biosensors or potent antibiotics. Recently, William Wimley et al. have developed an effective method to rationally engineer and screen activity of MPPs.\textsuperscript{9, 10, 23} 

Due to the fact that there is little to no sequence homology for AMPs, it proves to be a very taxing effort to engineer effective MPPs without having a sound starting point. Wimley’s design, however, gives rise to a rational combinatorial design of MPPs, based on the composition of the peptides rather than the sequence. Taking this into account, Wimley is sure to include aliphatic residues into the peptides for easy insertion into the hydrophobic interior of the bilayer. Additionally, aromatic residues are incorporated into the sequence, as they are highly prevalent in the membrane interface region of membrane active proteins.

The skeleton used for the combinatorial design is composed of a nine-residue core. This is an ideal length to use, as it is short, keeping the complexity of the design as simple as possible. Further, while nine-residues constitute a fairly short peptide, it is long enough to function; there are well-characterized AMPs of this length that act as effective pore-formers. Within the nine-residue core, there are five positions that were fixed with hydrophobic residues. These five hydrophobic residues, located at every other position in the core, act in two ways: first, they limit the complexity and the breadth of the combinatorial library; secondly, they provide a hydrophobic driving force for the peptide to interact with the membrane, essentially making the peptide much more likely to have pore-forming activity. At the two ends of the core are aromatic residues, either tryptophan or tyrosine. These residues have two roles: not only do they add to the overall
hydrophobicity of the peptide, but also account for the aromatic interactions that are so prevalent among membrane proteins.

Located between the five fixed positions is one of eight variable residues. This creates a dyad repeat of polar/nonpolar residues in the core, a common motif among many β-sheet structures within membranes. In theory, this allows for one face of the peptide to interact with the hydrophobic interior of the membrane, leaving the polar face of the peptide freely exposed to water. The four variable sites are occupied by one of eight possibilities. Four of the eight possibilities are the polar or charged residues of threonine, arginine, aspartic acid or asparagine. The other half of the possibilities are from a collection of hydrophobic residues: glycine, alanine, valine or tyrosine. Further variations occur at the termini of the peptides. The basic cassette of RRG or GRR (with the R always at the terminal position) could occur at both, either or neither of the termini. The role of the basic cassette is to increase the solubility of the already hydrophobic peptides by adding charged residues to the ends.

\[(RRG)-W-X-L-X-L-X-L-Y-(GRR)\]

A library was compiled, consisting of all possible variations of the peptides using the split and pool method of synthesis. This method allows for a randomized sequence in the creation of a 16,384-member combinatorial library of rationally designed MPPs.

With such an expansive compendium of peptides, it is necessary to establish an effective and efficient high-throughput assay in order to screen the MPPs for pore-forming activity. First, it is essential to create a model system for a biological membrane.
Large unilamellar vesicles (LUVs or liposomes) are prepared to act as an experimental model. The liposomes are encapsulated with terbium (Tb$^{3+}$) and DPA, an aromatic chelator, exists freely in solution. Upon chelating Tb$^{3+}$, a highly luminescent Tb$^{3+}$/DPA complex forms. This luminescence is easily detectable with a fluorescence spectrophotometer, allowing for an accurate and timely collection of data over time. An increase in fluorescence is directly correlated to peptide-induced leakage of the LUV. The peptides are added to the liposome solution and the increase in fluorescence is monitored over time. At the time a peptide from the library is added to the liposome, its sequence was unknown. Thus, peptides that showed activity as pore-formers were retroactively sequenced.

In their 2008 studies, Wimley et al. found RRGWVLALYLRYGRR (VAYR), among others, to be active in the membrane. This peptide induces a significant amount of leakage, at a measurable rate, compared to the rest of the peptides in the library. This peptide, as well as the other 11 effective peptides, was characterized by circular dichroism (CD) to be a β-strand structure. It should be noted that these 11 peptides are not as highly ordered as other well-characterized AMPs. Due to their short length, it is highly unlikely that they form intramolecular β-sheets, but rather the interactions are intermolecular.

While it is not completely determined, Wimley was able to infer a substantial amount about the mechanism of pore-formation for his series of effective MPPs. The results of the leakage experiments consistently suggest that the MPPs induce an “all-or-none” mechanism of leakage. This suggests that upon being treated with the peptides, a
portion of the liposomes release all of their contents, while the other portion releases none of their contents.

Further, the study suggests that the pore formation follows the “carpet” (also known as “sinking raft”) model. This model, as well as the studied MPPs, function by forming a transient pore. Additionally, both the model and the studied MPPs most likely are driven by the interfacial activity of the peptides. In this carpet model, peptides initially bind to the outer monolayer of the lipid surface. The peptides are then able to self-assemble on the membrane interface, creating an imbalance of charge, mass or surface tension. Following this, transient pores forms in the bilayer, relieving the stresses imparted onto the membrane from the binding of the peptides. The pores induce leakage of the liposomal contents before the peptides equilibrate between the vesicles, essentially closing the pore.

Overall, the experimental procedure developed by Wimley et al., proves to be a very effective and efficient way to study the interactions between peptides and membranes. These studies served as a template for our investigations, using the VAYR series as the model system. This shows measurable activity in terms of pore-formation, and has been extensively characterized by Wimley, and also proved to be very dependable for providing repeatable results. Although these peptides were discovered by a rational design, the intricacies of the mechanism MPP activity are poorly understood. Further studies need to examine the nuances of this complex system in order to achieve a fuller understanding of the interactions between proteins and membranes.
2.5 Using Fluorinated Amino Acids as Probes

One of the most commonly used methods of studying a complex system, such as protein—membrane interactions, is to incorporate mutations into a well-known system and observe the differences. It is exceedingly important that the mutant is as similar to the model system as possible. This refines the number of variables associated with the experiment, allowing for an easier interpretation of data. By introducing mutations into the amino acid sequence of a protein, many characteristics of the protein and the role of the individual amino acids can be illuminated, and even exploited. Once understood, the mutations can be directed towards the design of a particular structure, often with increased stability or functional activity. Although incorporating mutant amino acids into a sequence is a small change, the perturbation to the model can be reduced even further. Instead of having a complete one-residue mutation on the system, the mutation can be as small as one single atom with the use of unnatural amino acids. This type of mutation is particularly useful in determining the nuances and intricacies between the structure—function relationship of proteins.

The use of fluorinated amino acids is a very effective and widely used method to study the detailed structure—function relationship of proteins. More importantly, fluorinated amino acid analogs have been proven to have capabilities that hyper-stabilize protein structure, implicating them in a multitude of potential uses for protein design.²⁴

Foremost, it is necessary to understand the properties of fluorine that make it such an effective element as a probe in protein systems. While fluorine is an extremely abundant chemical on Earth, it very rarely occurs within biological systems. One of the most advantageous aspects of using fluorine in protein systems is its comparable size to
hydrogen. Thus, it can be readily substituted for hydrogen, while resulting in minimal
perturbations to the system in terms of its steric bulk. Another favorable characteristic of
the use of fluorine is the super-hydrophobicity of fluoro-carbons. This can be especially
useful in protein studies, as hydrophobicity is an important driving force in protein
folding. Hence, if exploited properly, a dramatic increase in the hydrophobicity of
certain parts of a protein would increase the stability of the final folded conformer.
Another unique advantage to fluorination is the potential “fluorous-effect,” which drives
the self-association of fluorinated moieties. Again, this proves to be useful in protein
studies, and if tailored precisely, this property could enhance the way in which proteins
interact both inter- and intramolecularly. An additional factor associated with the use of
fluorinated amino acid analogs has to deal with the intrinsic property of fluorine’s
electronegativity. Fluorine is the most electronegative element on the periodic table, so
incorporating a fluorinated moiety would greatly enhance inductive effect on a molecule.
This is an immensely valuable tool, as this property, too, can be paramount in the
engineering of proteins. Taken together, these properties attest to the subtly unique and
powerful potential of fluorinated amino acids.

To date, there has been a substantial amount of research, which integrates
fluorinated moieties into aliphatic amino acids. The utilization of fluorinated aromatic
residues, however, has received much less attention. Many of the studies have shown a
direct correlation between the addition of fluorinated moieties and the increased stability
of the protein structure.\textsuperscript{25-28} However, it is important to note that the addition of
fluorination does not absolutely result in increased stability. It is truly based on the
context in which the fluorination is used.
In his study based upon fluorinated aromatic amino acids, Gellman et al. illuminated the potential, as well as the restrictions, of using fluorination to increase the stability of peptides. The studies revolved around using HP35 as a model system. HP35 is a 35-residue subdomain of the chicken villin headpiece. This subdomain consists of three Phe residues in the hydrophobic core that share close proximity to one another. The system was probed with the incorporation of pentafluoro-phenylalanine into the hydrophobic core. Of the seven different mutations that were studied, only one of the mutations increased the stability of the protein. These findings insist that the mutations must be carefully designed in order to increase the stability of the protein, and that there are a plethora of interactions that account for the overall stability. Protein stabilization is a very detailed, intricate process, making favorable deviations from the wild-type conformation difficult to come across without careful thought. Regardless, the fact that a more stable conformation of the protein was found does indicate the capacity for fluorinated amino acid analogs for protein engineering. Further, this HP35 system proves to be an ideal system for studying aromatic interactions in the hydrophobic core of a protein.

2.6 Goals of this Study

The study of fluorinated, aromatic amino acids remains a largely unexplored prospect in this field. Our studies first undertake the design and synthesis of novel amino acids. As previously explained, the use of the unnatural amino acids provides insight into natural and de novo proteins that the canonical 20 natural amino acids do not. We aim to
first create a high-yield synthesis route of both 2,3,4,5-tetrafluro-L-phenylalanine (Z₀) and 2,3,5,6-tetrafluro-L-phenylalanine (Zₚ).

Once synthesized, these amino acids will serve as a probe to several protein systems. First, we seek to strategically integrate these amino acid derivatives into a well-characterized protein system, such as HP35. We suspect that they will interact differently than pentafluoro-phenylalanine (Z), which Gellman used, as these residues maintain an aromatic hydrogen that was not accounted for in the previous studies.

Next, we will focus our efforts into incorporating Z₀ and Zₚ into MPP systems in order to explore their affinity for enhancing pore-formation in biological membrane mimics. We believe that incorporating aromatic, fluorinated residues will enhance the protein—membrane interaction at the interface region, as aromatic residues are focused in this region in native proteins. Through this, we hope to gain a fuller understanding of how fluorinated moieties can interact with membrane systems. We keep in mind the long-term goal of being able to harness the power of fluorination to bring us closer to the development of novel, potent antibiotics, capable of avoiding bacterial resistance—especially in an era where bacterial resistance to our most commonly used antibiotics poses an imminent threat to the infrastructure of our primary care strategy.
3. MATERIALS AND METHODS

3.1 Synthesis of $Z_o$ and $Z_p$

The tetrafluoro-L-phenylalanine derivatives were originally synthesized according to the following reaction scheme (Scheme 1).

**Scheme 1**

This reaction scheme and molecular characteristics are reported in detail in our previous publication. An alternative route of synthesis follows:

**Scheme 2**
**Synthesis of Chiral Auxiliary, 7**

In a round-bottom flask, 3.7 eq of tritylloxonium-tetrafluoroborate was dissolved in anhydrous DCM. 1 eq of 6 was slowly added to the reaction vessel. The reaction occurred while stirring at room temperature for 72 hours. The reaction was quenched with ice water and 14% ammonia solution. The reaction was extracted with DCM and the organic layer was washed with saturated NaCl solution and water (1:1). The product was dried over sodium sulfate. The product was purified on a silica gel column with a solvent system of 10:1 hexanes:ethyl acetate, $R_f=0.3$, TLC plates stained with KMnO₄. The reaction proceeded with 74% yield.

**Alkylation, 8**

Under argon, 1 eq of the chiral auxiliary was dissolved in dry THF and cooled to -78°C. Deprotonation was carried out by slowly titrating 1.2 eq of nBuLi into the reaction, allowing it to react for 30 minutes. Next, 1.2 eq of tetrafluoro-benzyl bromide in dry THF was slowly added to the reaction. The reaction was monitored by TLC, 10:1 hexanes:ethyl acetate until completion, which occurred after 1.5 hours. The reaction was quenched with saturated NH₄Cl. The organic layer was separated and the aqueous layer was extracted three times with DCM. The combined organic layers were washed three times with brine and dried with magnesium sulfate. The product was purified on a silica column using 1:1 DCM:hexanes, and then pure DCM one the side product passed through. The product had an $R_f$ of 0.2, and was collected for an overall reaction yield of 84%. $^1$H-NMR 400MHz, $\delta=6.83$ (m, 1H), 4.10-3.89 (m, 5H), 3.67 (m, 1H), 3.25 (dd, 1H), 2.90 (dd, 1H), 2.15 (m, 1H), 1.20-1.16 (m, 3H), 1.13-1.10 (m, 3H), 0.91-0.90 (m,
3H), 0.59-0.57 (m, 3H). $^{13}$C-NMR 400MHz, δ=163.94, 162.16, 146.97, 144.52, 118.67, 104.06, 60.96, 55.02, 32.07, 28.99, 19.26, 16.89, 14.46. HR-MS calculated for C$_{18}$H$_{23}$F$_{4}$N$_{2}$O$_{2}$ [M$^+$] 375.1696, found 375.1693.

**Acid Hydrolysis, 9**

The alkylation product was dissolved in THF at rt and cooled to 0˚C. Slowly, 2N HCl was added to the solution, and it was allowed to react while warming to room temperature for 2 hours. Excess ice was added to quench the reaction. The pH was adjusted to 8 with saturated NH$_3$. The organic layer was separated and the aqueous layer was extracted three times with DCM. The organic layers were combined and dried with magnesium sulfate. The product was purified on a silica gel column, using ethyl acetate. The product was monitored on TLC, stained with ninhydrin solution, and had an R$_f$ of 0.8, and was collected for an overall reaction yield of 89%. $^1$H-NMR 400MHz, δ=6.89-6.87 (m, 1H), 4.10-4.05 (m, 2H), 3.63-3.59 (m, 1H), 3.06 (m, 1H), 2.89 (m, 1H), 1.16-1.01 (m, 3H). $^{13}$C-NMR 400MHz, δ= 104.698, 77.214, 61.544, 54.118, 28.769, 14.228. HR-MS calculated for C$_{11}$H$_{12}$F$_{4}$NO$_{2}$ [M$^+$] 266.0804, found 266.0798.

**Base Hydrolysis, 4**

The acid hydrolysis product was dissolved in ethanol, and reacted with 1M NaOH at 50˚C for 2 hours, monitored by TLC. Upon completion, the reaction was quenched with ice and the pH of the solution was adjusted to 7 with 2N HCl. The product was not purified, and 100% yield was assumed for the next step.
The amino acid was dissolved in 9% sodium carbonate and cooled to 0°C. 0.95 eq of Fmoc-OSu was dissolved in DMF and cooled to 0°C and slowly added to the amino acid solution. The reaction proceeded for 30 minutes at room temperature. The reaction was then diluted with water, and extracted first with diethyl ether, then ethyl acetate, twice. The aqueous phase was tuned to pH 2 with concentrated HCl to push the product into the organic phase. The aqueous phase was then extracted with ethyl acetate six times. The solution was washed with saturated NaCl solution three times and then water twice. The product was purified by silica gel filtration in a 3:1:4% DCM:hexanes:acetic acid solvent system. The R_f value of the product was about 0.2, and the product was collected for an overall reaction yield of 79% yield.

3.2 NMR Spectroscopy

Organic Synthesis

The product from each step of the reaction was characterized by both ^1^H-Nuclear Magnetic Resonance Spectrometry (NMR) and ^1^C-NMR. The NMR data was collected using the Varian Gemini 400MHz NMR Spectrometer. All products were dissolved in CDCl_3. The chemical shifts from the spectra are recorded in ppm.

HP35 Variants

The HP35 peptide variants were prepared for 1D ^1^H-NMR in a 20mM sodium phosphate buffer, pH 5.5, H2O:D2O 9:1 (v/v), with the total peptide concentration at 1mM. The 1D ^1^H-NMR data was collected on a Varian INOVA 500MHz NMR
spectrometer. WATERGATE pulse sequences were utilized to suppress the water peak. The NMR data were processed by the MestRe Nova software (Mestrelab Research S.L., Spain), and the residual solvent peak was suppressed using digital suppression.

### 3.3 Peptide Synthesis

*Synthesis*

All peptides were synthesized via automated solid phase peptide synthesis, utilizing Fmoc protection chemistry on a Tribute Peptide Synthesizer (Protein Technologies, Tucson, AZ). The peptides were synthesized most often on a 0.1 mmol scale, using five-times excess equivalents of each of the Fmoc-protected, commercially available amino acid residues (Advanced Chemtech, Louisville, KY). Fmoc-Wang resin (Novabiochem) was used as the solid support for the synthesis. The resin was washed and swollen with dimethylformamide (DMF) for 20 minutes. The resin was then deprotected with 20% piperidine in DMF, for 5 minutes, two times. The amino acids were activated with slightly less than one equivalent 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyllumonium hexafluorophosphate (HBTU). The amino acids were coupled by aliquoting the sequential amino acid/HBTU into the resin, which reacted under 3 mL DMF and 0.4 M N-methylmorpholine for 2.5 minutes. The resin was washed with DMF to remove all excess amino acid and HBTU. The amino acid was then deprotected with 3 mL piperidine solution. The cycle continued like this until the end of the sequence. At the end of the sequence, a final Fmoc deprotection was initiated.

Often, the unnatural amino acids were manually coupled to the sequence. During this process, only two-times excess was used, and coupling time was doubled. Also,
often when an Arg residue was in the sequence, the coupling time was doubled in order to help combat the issue of poor Arg coupling.

Cleavage

Peptides were cleaved from the resin and their protecting groups were removed with Reagent K (80% TFA, 5% H₂O, 2.5% ethanedithiol [EDT], 5% thioanisole and 7.5% phenol) for the HP35 studies. Reagent R (90% TFA, 5% thioanisole, 3% EDT, 2% anisole) was used to cleave the MPPs. The reagent cocktail stirred with the resin at room temperature for one hour, and the supernatant was drained off. The peptide was precipitated from the solution by extraction with cold diethyl ether. The solution was then centrifuged, the supernatant was drained, and the cycle was repeated three times to wash.

3.4 High Performance Liquid Chromatography

High Performance Liquid Chromatography

The peptides were purified via high performance liquid chromatography (HPLC). The instrument was a Waters PrepLC System, using a C18 300A column (Waters Corporation, Milford, MA). A gradient of two buffers was established for separation. Buffer A consisted of 95% water, 5% acetonitrile and 0.1% TFA and Buffer B consisted of 95% acetonitrile, 5% water and 0.1% TFA. The gradient began at 100% A, while moving to 80% A (20% B) over the course of 6 minutes, then to 50% A over the course of 5 minutes, and then to 5% A over 10 minutes, and finally the column was washed with 100% A for 10 minutes. The gradient was tweaked on a case-by-case basis, depending
on the properties of the peptide. The eluent was monitored by an ultraviolet and visible (UV-vis) spectrophotometer, set to dually monitor at 220 nm for the peptide backbone and 280 nm for the Trp residue. Fractions were manually collected as peaks appeared on the UV-vis readout.

_Fast Protein Liquid Chromatography_

The HP35 and variants thereof were further purified through fast protein liquid chromatography (FPLC). The HP35 variants were subjected to gel filtration, using a UPC-900 system with a HiLoad 16/60 Superdex™ 30 prep-grade column (General Electric, Piscataway, NJ).

3.5 _Lyophilizer_

All peptides were dried completely by lyophilization overnight. The peptides were placed in a minimal amount of aqueous solution, and frozen before being placed on the Freezemobile 25ER Sentry 2.0 lyophilizer (VirTis, Gardiner, NY).

3.6 _Mass Spectrometry_

Samples, both organic products and peptides, were characterized at the Mass Spectrometry Center at Boston College. High-resolution mass spectrometry (MS) was used to characterize each of the products from the organic syntheses. Electron spray ionization (ESI) or MALDI was used to characterize the peptides. Liquid chromatography/mass spectrometry (LC/MS) was also used to characterize the peptides.
The LC/MS gradient was 95% water, 5% acetonitrile, 0.1% formic acid to was 5% water, 95% acetonitrile, 0.1% formic acid over 20 minutes.

3.7 Circular Dichroism

Circular dichroism (CD) was utilized to determine secondary structure, thermal melting data and denaturation curves by guanidinium chloride (Gdm·Cl) for the peptide systems. The CD spectrometer was an AVIV 202SF (Biomedical, Inc., Lakewood, NJ), with a temperature-controlled cell holder, utilizing a 2mm quartz cell.

Secondary Structure

The peptides were subject to a wavelength scan to determine their secondary structure. A 50 µM protein solution was prepared in a buffer of 20 mM Na·Pi, 150 mM NaCl, pH 7.0. The sample was subjected to a wavelength scan ranging from 195 to 260 nm at 2˚C.

Thermal Melting

Thermal melting experiments were acquired by monitoring a constant wavelength of 222 nm over a temperature course of 2˚-102˚C. The temperature increases were performed in 2˚ increments, allowing 90 seconds for equilibration time. The signals were averaged over 30 seconds. Once the temperature reaches a maximum, a reverse scan was obtained, thus both denaturation and renaturation tendencies were accounted for.
**Guanidinium Chloride Denaturation**

Gdm·Cl denaturation experiments were observed at 2°C and monitored at 225. A 10 µM solution of peptide in its native buffer (20 mM Na·Pi, 150 mM NaCl, pH 7.0) was titrated with the denaturing buffer (20 mM Na·Pi, 150 mM NaCl, 7M Gdm·Cl, pH 7.0). The denaturing buffer was added in fractions, and two minutes of equilibration time were allowed between each addition; a stir bar in the cuvet mixed the solution. Before the addition, an equal portion of the solution was removed, so that the total volume was constant throughout.

### 3.8 Liposome Preparation

Liposomes were prepared by mixing 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) and 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoglycerol (POPG) in a 9:1 POPC:POPG ratio (Avanti Polar Lipids, Alabaster, AL). The lipids were dissolved in chloroform and thoroughly mixed. The chloroform was evaporated, leaving a homogenous mixture of lipids. The lipids were then hydrolyzed in a 100 mM triethylsilane (TES), 10 mM sodium citrate, 50 mM TbCl₃ buffer. Full encapsulation and vesicle formation was ensured by performing 20 freeze-thaw cycles, in which the mixture was frozen at -70°C and then thawed at 80°C. Next, the lipids were extruded through an extruder (Avanti Polar Lipids) using a 100 nm filter to ensure for uniformly sized liposomes. The liposomes were passed through the extrusion filter 15 times. The liposomes were then subjected to gel filtration, using a UPC-900 system with a sephacryl S-500 HR column (General Electric, Piscataway, NJ) under a 100 mM TES, 10 mM sodium citrate (TES buffer) eluent. The eluent was monitored by UV-vis
spectrophotometry, and a 3 mL peak region was collected. The resulting liposome stock solution existed at a final concentration of 12 mM.

3.9 Leakage Assays

The leakage assays were monitored on a fluorimetry spectrophotometer. Time dependent studies were performed using excitation/emission wavelength parameters at 270/489 nm, respectively. Leakage was measured by an increase in fluorescence upon the chelation of Tb$^{3+}$ by DPA. A solution of TES buffer with 480 µM liposome and 50 µM DPA was placed in a 1 cm x 1 cm x 2 mm quartz cuvet. The fluorimeter monitored this baseline reading for 200 s, at which time 300 µM peptide in TES buffer and 8 M Gdm·Cl was titrated into the cuvet, pipetted up and down in order to ensure complete mixing. The leakage was monitored for 550 more seconds, at which time 60 µL of 1% Triton X detergent was added to the solution to induce complete leakage, to serve as the maximum value of leakage.
4. RESULTS

4.1 Incorporating Z_o and Z_p mutants into HP35

The data obtained through NMR, thermal denaturation and Gdm·Cl denaturation studies show that the stabilities of the peptides, in increasing order of stability, is $F6Z < F6Z_p < F10Z_p < WT < F10Z < F10Z_o$. This indicates that fluorination can be used to increase the stability of proteins.

NMR Studies

Each of the HP35 variants was subjected to $^1$H-NMR analysis to determine any structural differences between them. The NMR spectra in Figure 1 display the chemical shifts for the WT, $F6Z_p$, $F10Z_o$ and $F10Z_p$ HP35 variants. The WT spectra displays two upfield shifted resonances at 0.10 ppm and 0.46 ppm, which are assigned to the $H^7$ of Val9 and the $H^8$ of Leu20, respectively. This, along with the nearly identical patterns in the chemical shifts, asserts that there are very few differences between the spectra of the variants.
**Figure 1.** 1D $^1$H NMR Spectra of WT HP35 and Fluorinated Mutants

![NMR Spectra](image)

*Figure 1 displays the 1D $^1$H NMR spectra of WT HP35 and the fluorinated mutants. All mutants display well dispersed resonances and similar patterns to the WT.*

**Circular Dichroism to Determine Structural Propensities**

Each variation of HP35, including the WT, was subjected to CD spectrometry. Ellipticity measurements were monitored over a wavelength scan from 195-260 nm. Each variant of the peptide exhibited minima at 222 and 208 nm, which is the characteristic trace of an $\alpha$-helix. Each of the traces is nearly identical to that of the WT. The data is represented in Figure 2.
**Figure 2.** Wavelength Scan of HP35 Variants to Determine Secondary Structure

*Figure 2 displays the results of a wavelength scan of HP35 and the fluorinated mutants, via CD. The ellipticity minima at 222 and 208 nm are characteristic of an α-helical structure.*

**Thermal Denaturation**

The thermal stability of each of the HP35 mutants was analyzed through a series of thermal melting studies. Using CD, the ellipticity was be monitored at 222 nm to determine the melting temperature ($T_m$) of each of the variants. The thermal melting data, tabulated in Figure 3 displays a two-state model, and the (un)folding was shown to be completely reversible. The thermal stability of the mutants increases from $F6Z < F6Z_p < F10Z_p < WT < F10Z < F10Z_o$. The data is tabulated in Table 3.
Figure 3. Thermal Melting studies on HP35 and Variants

Figure 3 shows the thermal stability of HP35 and the fluorinated mutants. (A) The (un)folding conforms to a two-state model. (B) The melting curves of each of the HP35 variants.

Gdm·Cl Denaturation Studies on HP35 and Variants

Gdm·Cl denaturation studies, shown in Figure 4, were performed to determine the folding free energies ($\Delta G_f$) of each of the HP35 variants. The data is consistent with the thermal melting data, showing that the peptides increase in stability from $F6Z < F6Z_p <$
\(F_{10Z_p} < WT < F_{10Z} < F_{10Z_o}\). From the curves, \(\Delta G_f\) was extrapolated, and the results are tabulated in Table 3.

Figure 4. Gdm·Cl Denaturation to Determine \(\Delta G_f\) Values

The Gdm·Cl denaturation curves provide a way to measure the folding free energies (\(\Delta G_f\)) of each of the HP35 variants. A linear fit of the data (B) gives the exact value of \(\Delta G_f\), which is summarized along with all other thermodynamic data in Table 3.
4.2 Membrane Pore-forming Peptides

Overall, selective mutations to fluorinated amino acids were capable of enhancing the pore-forming activity of the MPPs. The results indicate that the position of the mutation plays a role in its efficacy, implying that the enhanced activity is not due a single factor, such as hydrophobicity, but is a more complex system.

Mutant Peptides Studied

In total, 16 peptides were synthesized and purified for extensive testing. The peptides, summarized in Tables 1 and 2, followed the WVAYR series. The mutations were incorporated in one of two positions, so that the variable peptide series followed the VAXR* or XVAFR sequences.

<table>
<thead>
<tr>
<th>Table 1. XVAFR Mutant Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
</tr>
<tr>
<td>YVAFR:</td>
</tr>
<tr>
<td>FVAFR:</td>
</tr>
<tr>
<td>ZVAFR:</td>
</tr>
<tr>
<td>Z₀ VAFR:</td>
</tr>
<tr>
<td>Zₚ VAFR:</td>
</tr>
<tr>
<td>Z₃₄₅F VAFR:</td>
</tr>
<tr>
<td>Z₃₄₅F VAFR</td>
</tr>
</tbody>
</table>

Table 1 shows the XVAFR mutant peptides. Each peptide contains a single mutation in place of the wildtype Trp residue.

* VAXR is the shorthand for WVAXR
Table 2. WVAXR Mutant Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Complete Peptide sequence</th>
<th>Mutation (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WVAYR (WT)</td>
<td>RRGWVLAL-Y-LRYGRR</td>
<td>WT</td>
</tr>
<tr>
<td>WVAFR:</td>
<td>RRGWVLAL-F-LRYGRR</td>
<td>F</td>
</tr>
<tr>
<td>WVAZR:</td>
<td>RRGWVLAL-Z-LRYGRR</td>
<td>Z</td>
</tr>
<tr>
<td>WVAZₜₕR:</td>
<td>RRGWVLAL-Zₜₕ-LRYGRR</td>
<td>Zₜₕ</td>
</tr>
<tr>
<td>WVAZₚₜR:</td>
<td>RRGWVLAL-Zₚₜ-LRYGRR</td>
<td>Zₚₜ</td>
</tr>
<tr>
<td>WVAZ₃₄₅FₜR:</td>
<td>RRGWVLAL-Z₃₄₅Fₜ-LRYGRR</td>
<td>Z₃₄₅Fₜ</td>
</tr>
<tr>
<td>WVAZ₄₅₆FₜR:</td>
<td>RRGWVLAL-Z₄₅₆Fₜ-LRYGRR</td>
<td>Z₄₅₆Fₜ</td>
</tr>
<tr>
<td>WVAChₜₕR:</td>
<td>RRGWVLAL-Cₜₕ-LRYGRR</td>
<td>Cₜₕ</td>
</tr>
<tr>
<td>WVAWR:</td>
<td>RRGWVLAL-W-LRYGRR</td>
<td>W</td>
</tr>
</tbody>
</table>

Table 2 shows the WVAXR mutants. Each peptide contains a single mutation for the original Tyr residue.

Binding Affinity of MPPs

In order to determine whether or not the mutations enhanced the binding of the peptides to the membrane, two peptides, VAYR and VAFR were titrated with liposomes (Figure 5). The experiment was monitored by fluorescence enhancement of Trp at 330 nm, as the peptides bound onto the liposomes. The data indicates that both peptide series become saturated with lipid at the same concentration. The concentration of lipid, 40 µL, puts the peptide:lipid ratio at 1:20. This indicates that the peptides are completely bound to the liposome when the concentration is at least 1:20.
**Figure 5.** Binding Affinity of MPPs to the Liposomes

![Graph showing binding affinity](image)

*Figure 5 shows the binding affinity of the MPPs to the liposomes. The data indicates that the peptides are completely saturated with liposomes when the peptide:lipid ratio is 1:20.*

**Leakage Assays for VAXR and XVAFR Peptide Series**

The peptides in Tables 2 and 1 were assayed to assess their ability to induce leakage in the liposome system. Figures 6 and 7 contain all the results of the leakage experiments, which were all carried out under the same conditions. For the VAXR series, in Figure 6, the most potent peptides, in decreasing order, were: VAZR > VAZpR > VAZoR > VAChaR > VAZ345F-R > VAYR(WT) > VAZ4F-R > VAFR. Figure 7 depicts the results from the XVAFR series. The most effective peptides in this series in decreasing order were: ZVAFR > Z345F VAFR > Zp VAFR > WVAFR(WT) > Z34F VAFR > YVAFR > FVAFR.
Figure 6. Leakage Assay of VAXR Series

![Figure 6. Leakage Assay of VAXR Series](image)

The efficacy of each of the peptides in the VAXR series to induce leakage from the liposome.

Figure 7. Leakage Assay of XVAFR Series

![Figure 7. Leakage Assay of XVAFR Series](image)

The efficacy of each of the peptides in the XVAFR series to induce leakage from the liposome.
Secondary Structure of MPPs

The relationship between the structure of the peptides and their pore-forming capabilities was studied by performing wavelength scans of two peptides, VAFR and VAZR. While VAZR is a more potent MPP, it displays the same level of β-sheet propensity as the less active VAFR (Figure 8). This data suggests either that the secondary structure of the peptide is not completely indicative of the pore-forming activity.

Figure 8. Secondary Structure of VAFR and VAZR

![Graph showing the secondary structure of VAFR and VAZR.](image)

Figure 8 shows the secondary structure of VAFR and VAZR. Both peptides exhibit an equal minimum at 218 nm, characteristic of a β-sheet secondary structure.

Self-Assembly of MPPs

AMPs and other MPPs have been implicated in possibly having the property of first self-assembling before inserting into the membrane. The MPPs studied here form β-sheet structures and may assemble before partitioning into the membrane. This is an analogous mechanism of action for amyloid proteins. To begin to test whether or not the
MPPs have amyloid tendencies, a binding assay of VAFR and VAZR to thioflavin-T (ThT) was performed. The results indicate that VAZR has a higher binding affinity to ThT than VAFR.

**Figure 9. Binding Assay of VAFR and VAZR to ThT**

![Binding Curves](image)

*Figure 9 shows the binding curve of VAFR and VAZR to ThT. The curves indicate that both peptides bind ThT, but VAZR has a significantly higher binding affinity than VAFR.*
5. DISCUSSION

5.1 Fluorination to Stabilize Protein Structure

Structure of HP35

The reason HP35 was chosen as the model system for this experiment is because of the structure of the peptide. HP35 is a well-characterized \( \alpha \)-helical system, which proves to be easy to study because of its clearly defined secondary structure. Moreover, the primary structure of the peptide provides a perfect backdrop to study how aromatic interactions can stabilize a protein fold. At the hydrophobic core of HP35, there are three phenylalanine residues in close enough proximity to interact with one another, as shown in Figure 10.

Figure 10. Cartoon Representation of HP35 Structure

A cartoon representation of the three Phe residues at the hydrophobic core of HP35 (PDB: IYRF). Hydrogen atoms engaging in ArH---\( \pi \) interactions are shown cyan.
Figure 10 gives insight into the aromatic interactions at the hydrophobic core of the protein. One prevalent interaction between the aromatic residues is the packing of aromatic hydrogens (ArH) into the $\pi$-electron cloud of the aromatic system. These ArH···$\pi$ interactions seem to be the most apparent mode of interaction between the Phe residues in HP35. As illustrated in Figure 10, it is clear that the H-4 of F6 packs into the $\pi$-cloud of F17, while the H-6 of F10 packs into the $\pi$-cloud of F6. The ArH···$\pi$ interaction between adjacent Phe residues is also depicted in the structural representation in Figure 11.

**Figure 11. Structural Representation of an ArH···$\pi$ Interaction**

![Image of ArH···$\pi$ Interaction]

*Figure 11 shows how an ArH (teal) can pack into a neighboring phenyl ring (green) to create an ArH···$\pi$ interaction.*

**Probing HP35 with Fluorinated Mutants**

Using the fluorinated amino acids $Z_o$ and $Z_p$ as probes to study protein stability is a multifaceted approach. First, it is known that fluoro-carbons are extremely hydrophobic, and increasing the hydrophobicity of residues in the hydrophobic core may
enhance protein stability. However, the fluorination of the Phe residues in the core of HP35 needs to be a selective design. As shown above, the ArHs of the Phe are believed to have a critical role in aromatic-aromatic interactions and protein stabilization as a whole. Thus, fully fluorinated residues that lack any ArHs may detract from the overall protein stability. To account for this, the use of *highly but not fully* fluorinated side chains was employed in hopes of increasing the stability of HP35.

Using *highly but not fully* fluorinated residues, such as Z₀ and Zₚ prove to be advantageous for many reasons. These fluorinated amino acid analogs have increased hydrophobicity, which could prove to enhance the interactions between aromatic residues in the hydrophobic core of HP35. Further, these unnatural amino acids may be able to improve the ArH···π interactions. Not only do they retain the necessary aromatic hydrogen atoms to engage in the interactions, but the fluorination will also drain electron density from the aromatic ring, leaving a the ArH with a higher partial positive charge. The electron deficient π-cloud will be more inclined to accept the hydrogen. Further, an ArH with a higher partial positive charge will have an increased predisposition to donate itself towards a π-cloud. Taken together, we hypothesize that the characteristics of *highly but not fully* fluorinated amino acids will enhance the ArH···π interactions and accordingly will yield a more stable protein.

The Spartan calculations of the fluorinated residues confirm the speculations that incomplete fluorination will increase the overall partial positive charge of the remaining hydrogen atoms, as shown in Figure 12. The H-4 on Zₚ bears a partial positive charge of 0.202. This is more than double the charge on the H-4 of the WT residue (0.089). Further, the partial charge on H-6 of Z₀ (0.168) is significantly more positive than the
same hydrogen on the WT (0.108). Throughout the experiments, mutants with the pentafluoro-phenylalanine (Z) were used as controls. The fully fluorinated moieties have no aromatic hydrogens, and thus is unable to donate hydrogens in ArH···π interactions.

**Figure 12.** Space-Filling Models of Phe and its Fluorinated Analogs

![Space-Filling Models](image)

*The partial charge of each of the hydrogen and fluorine atoms on the aromatic system is provided on the structure representation of the residue. In the space-filling model, blue is representative of a positive charge and red represents a negative charge.*

**Structure of the Mutants**

It is important to ensure that the structure of the mutants is conserved, and also analogous to the WT. This ensures that there are as few variables as possible and that the mutants can be more directly compared to both each other and to the WT. The structural data obtained through both NMR and CD did, indeed, indicate that HP35 and all variants had a similar structure.
The NMR studies (Figure 1) show very similar spectra between all of the mutants and in comparison to the WT. All of the samples contain two upfield peaks, which are assigned to Val9 and Leu20. The upfield shift is a result of the residues packing against the aromatic side chains in the hydrophobic core of the protein. Since all of the variants exhibit this behavior, it is strongly suggestive that there is zero to minimal structural perturbations among the mutants.

This was further confirmed through a series of studies completed by CD (Figure 2). All of the samples exhibit ellipticity minima at 208 and 222 nm, which is characteristic of an α-helix. There are nearly identical traces for each of the variants, again suggesting the conservation of structure between the mutants in relation to the WT. Taken together with the NMR data, it is safe to conclude that there are minimal perturbations in the structure of the peptide through incorporating the tetrafluoro-phenylalanine residues into the hydrophobic core. This allows for apt and legitimate comparisons to be made to describe the effects of fluorination to protein stabilization.

**Stability of the Mutants**

In order to assess the stability of the mutants, they were each subjected to a series of thermodynamic analyses (Figures 2-4). The results of these studies are summarized in Table 3.
Table 3. Thermodynamic Properties of HP35 and Variants

<table>
<thead>
<tr>
<th>protein</th>
<th>$T_m^a$ (°C)</th>
<th>$C_m^b$ (M)</th>
<th>$m$ value$^b$ (kcal/(mol·M))</th>
<th>$\Delta G^b$ (kcal/mol)</th>
<th>$\Delta \Delta G_f^c$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>66</td>
<td>3.7</td>
<td>0.88</td>
<td>$-3.3 \pm 0.1$</td>
<td>0</td>
</tr>
<tr>
<td>F6Z</td>
<td>51</td>
<td>2.3</td>
<td>1.02</td>
<td>$-2.3 \pm 0.1$</td>
<td>+1.0 ± 0.1</td>
</tr>
<tr>
<td>F6Z$_p$</td>
<td>62</td>
<td>3.0</td>
<td>0.89</td>
<td>$-2.6 \pm 0.1$</td>
<td>+0.7 ± 0.1</td>
</tr>
<tr>
<td>F10Z</td>
<td>68</td>
<td>3.6</td>
<td>0.99</td>
<td>$-3.6 \pm 0.1$</td>
<td>-0.3 ± 0.1</td>
</tr>
<tr>
<td>F10Z$_o$</td>
<td>80</td>
<td>5.0</td>
<td>0.93</td>
<td>$-4.7 \pm 0.1$</td>
<td>-1.4 ± 0.1</td>
</tr>
<tr>
<td>F10Z$_p$</td>
<td>64</td>
<td>3.6</td>
<td>0.89</td>
<td>$-3.2 \pm 0.1$</td>
<td>+0.1 ± 0.1</td>
</tr>
</tbody>
</table>

The thermodynamic variants of HP35 (WT) and mutants thereof as determined through a series of thermal melting and Gdm-Cl denaturation experiments.

$^a$Thermal melting calculated by fitting thermal denaturation curves

$^b$Calculated by fitting GdmCl denaturation curves

$^c\Delta \Delta G_f = \Delta G_{f\text{mutant}} - \Delta G_{f\text{WT}}$

From the data, it is clear that the results from the thermal melting studies correlate directly to the Gdm-Cl denaturation studies. By first analyzing the control experiments, it is interesting to note that the F6Z mutant yielded opposite results than the F10Z mutant, with respect to the WT. The F6Z mutant is less stable than the WT by 15°C and also by 1 kcal/mol. In contrast, the F10Z mutant is slightly more stable than the WT by 2°C, or by an energy difference of 0.3 kcal/mol. A possible reason for this difference can be found by comparing the roles of the F6 and F10 residues. From the cartoon representation of HP35 in Figure 10, it seems that F10 acts more as a proton acceptor than anything else. Thus, the fully-fluorinated mutation is actually favorable at this position. The fluorination drains electron density from the phenyl ring, giving this residue a higher propensity to accept the partial positive charge of a neighboring ArH. On the other hand, the function of F6 in HP35 has a dual role of both a donor and acceptor. The H-4 of F6
packs neatly into the phenyl ring of F17, but the F6Z mutant is unable to perform this
duty without any ArHs. As a result, this mutant is less stable than the wildtype.
Therefore, F6 seemingly acts more as an ArH donor than an acceptor, and removing this
capability comes at the consequence of lower protein stability.

Comparing the control mutants with the tetra-fluorinated varieties gives useful
insight into the power of fluorination. F6Z_p is significantly more stable than the F6Z control. This increase in stability can be attributed to the regain of function for the H-4 to
pack into the π-cloud of F17. The F6Z_p mutant is 11˚C and 0.3 kcal/mol more stable than
the F6Z control.

The most astonishing results were from the F10Z_o mutant. F10Z_o is the most
stable peptide in the entire series. There is only a one atom difference between F10Z and
F10Z_o, yet F10Z_o is much more stable, by a factor of 12˚C or 1.1kcal/mol. The fact that a
single atom can account for such a large margin in stability is truly outstanding. This
increase in stability is indicative of the significance of the ArH···π interactions. It is
interesting to look into the results of F10Z_p, which proved to be 1.5 kcal/mol less stable
than the Z_o mutant, and also slightly less stable than the WT. This evokes upon the
theory that ArH···π are a leading factor in the stability of HP35. While F10Z_p and F10Z_o
are stoichiometrically identical, the F10Z_p lacks the necessary ortho-hydrogen to engage
in the ArH···π interactions.

There is an apparent difference between the ArH···π interactions at the F10 and F6
positions. The difference in stability between F6Z and F6Z_p is only 0.3 kcal/mol, while
the analogous difference between F10Z and F10Z_o provides a 1.1 kcal/mol increase in
stability. We postulate that this disparity is due to the fact that F6 is partially solvent
exposed. The electrostatic ArH···π interaction would be significantly weakened in the presence of solvent. F10, in contrast, is completely buried in the hydrophobic core of the protein. This would account for why the F10 ArH···π interactions are seemingly more significant than the same type of interactions at F6.

Summary

Evidently, it has been shown that fluorination can be used to increase the stability of a protein. Selective fluorination proves to be even more effective in creating a more stable structure. Our results insist that highly, not fully fluorinated amino acids are especially useful for enhancing the highly abundant ArH···π interactions of proteins. This method can be widely adapted for the design of hyperstable protein structures, as well as to enhance protein-protein driven interactions.

5.2 Using Fluorination to Design Effective Membrane Pore-forming Peptides

Wimley’s Model System and Selection of Mutants

The peptide series designed by Wimley et al. proves to be a very effective means of studying MPPs. One of the effective peptide series was VAYR, which was used as the model system for the bulk of our studies. The rational design of this peptide ensures that it adheres to the canonical structure of MPPs: there is an extensive hydrophobic core as well as an aromatic residue (Trp) in the interfacial region of the peptide, which together, can allow for a peptide to effectively partition into the membrane. By incorporating fluorinated amino acid mutations into both the hydrophobic core region and into the interfacial region, we hope to gain insight into the structure—function relationship of potent MPPs.
When selecting from a pool of possible mutations, many factors had to be taken into account, while in search for increased membrane activity. Incorporating fluorinated amino acids into a membrane model system is advantageous because they automatically provide an increase in hydrophobicity—an indispensable factor for membrane proteins. However, we believe that fluorinated moieties will contribute more to the system than just an increase in hydrophobicity. Fluorination can direct interactions between like proteins, which can aid in pore-formation activity. Further, *highly but not fully* fluorinated aromatic residues can provide a dipole interaction, which may prove to enhance the interactions at the membrane interface region.

Table 4 displays various characteristics, including the space-filling models and hydrophobicity (logP) of different fluorinated variants of phenylalanine, as well as other aromatic amino acid analogs. Phenylalanine as well as cyclohexane-alanine (C₇₈) will serve as controls. C₇₈ serves as an interesting control, as it displays the highest hydrophobicity (ClogP = 1.048), but it does not have an aromatic system. For the most part, molecules with a higher degree of fluorination, exhibited a higher degree of hydrophobicity than their respective counterparts. There are slight differences in the hydrophobicities of molecules of equal fluorination, i.e. Z₀ is slightly less hydrophobic than Z₁ (0.178 vs. 0.248 ClogP). The mutants outlined in Tables 1 and 2 were synthesized in order to decipher some of the principles of MPP activity.
Table 4. Hydrophobicities of Natural and Mutant Side Chains

<table>
<thead>
<tr>
<th>Log P</th>
<th>Log P*</th>
<th>Clog P*</th>
<th>Dipole (debye)</th>
<th>Surface Area (Å²)</th>
<th>Volume (Å³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.52</td>
<td>0.06</td>
<td>-0.184</td>
<td>0.26</td>
<td>132.53</td>
<td>103.31</td>
</tr>
<tr>
<td>2.68</td>
<td>0.22</td>
<td>-0.041</td>
<td>1.89</td>
<td>139.36</td>
<td>108.74</td>
</tr>
<tr>
<td>2.68</td>
<td>0.22</td>
<td>-0.041</td>
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The hydrophobicities of various side chains, in terms of Clog P terms. Dipole, surface area and volume calculations are also included in the table. *Chemdraw calculations with the N- and C- capped amino acids, AcNHCH-(R)CONH₂.
Binding Affinities

It was necessary for us to first determine whether or not fluorination affects the binding affinity of the peptides to the lipids. This will give insight into the intrinsic properties of fluorination and how it can affect the activity of MPPs. Figure 5 insists that the binding affinity of the peptides to the liposomes is not affected by fluorination. Both the fully fluorinated VAZR mutant and the VAFR peptide are fully saturated when the liposome concentration is at 40 µL. This data can be interpreted as the peptide is completely bound when the peptide/liposome ratio is at least 1:20. Above this concentration, the peptide binding, as measured by Trp fluorescence enhancement reaches a flat line, indicating complete saturation. In general, fluorination did not enhance the binding of the peptide to the liposome, which is an important factor to consider when comparing the membrane activities of different mutants.

Mutations in the Hydrophobic Core

The lytic activity of the peptides containing mutations in the core region was measured through a series of leakage assays, represented in Figure 6. Upon inserting into the membrane, the peptides induce leakage, allowing for Tb\(^{3+}\) to leak out of the liposome and form a highly luminescent complex with DPA. The monitored fluorescence is directly correlated to the extent of leakage caused by the MPPs. For the most part, the mutants in the VAXR series decrease potency following in the order of decreasing hydrophobicity. The pentafluoro mutant, VAZR is responsible for causing the highest extent of leakage, followed by VAZ\(_p\)R and then VAZ\(_o\)R. Of the fluorinated phenylalanine mutants, a lesser degree a fluorination resulted in a lesser extent of
leakage. It is worthy to note that although the series generally, but not completely,
followed the order of hydrophobicity. The C_{ha} mutant, which is the most hydrophobic
residue, caused an intermediate degree of leakage, falling between VAZ_{p}R and
VAZ_{345F}R. This finding suggests that while hydrophobicity does play an important role
in the pore-formation of MPPs, it is not the single, isolated factor.

*Mutations in the Interfacial Region*

In addition to strategically placing mutations in the hydrophobic core of the MPP
series, several mutations were made in the interfacial region of the MPP series, where a
Trp residue is usually located. By probing this location, we hope to gain insight into the
interactions at this region, which is highly conserved among many AMPs, but poorly
understood. The series of leakage experiments is graphically represented in Figure 7.
Again, the peptide that causes the most significant leakage is the pentafluoro mutant,
ZVAFR in this case. However, it is important to note that the W→Z mutation at the
interface region is not as significant a change as the Y→Z mutation in the core. Further,
it is worthy to mention that the order of the potency of the MPP mutants does not follow
the same pattern as it did for the VAXR mutants. The second most potent mutant was
Z_{345}VAFR, not the Z_{p} mutation as it had been in the VAXR series. Besides for the
pentafluoro mutant, the fluorinated residue mutations all induced a very similar amount
of leakage. This stands in contrast to the VAXR mutations, which were significantly
stratified in terms of their leakage efficiencies. Further, it seems that hydrophobicity at
this position does not play a paramount role in inducing leakage like it did in the VAXR
series.
A survey of the properties in Table 4 with regard to the membrane activities of the XVAFR class suggests that the polarity plays a critical role for MPP activity at the interfacial region. According to Table 4, Z and $Z_{345F}$ have the highest dipole of the unnatural amino acids incorporated into the MPP series. Upon consideration, it makes sense that a high dipole moment at the interfacial region would enhance the degree of leakage. The interface is a unique environment, partially solvent exposed. Thus, a residue with a high dipole would aid to the overall stability, easily interacting with both the polar environment and the hydrophobicity composing the membrane. If stable in its surrounding environment, MPP activity becomes more likely.

These findings bring about an interesting point, which is the relationship between the position of a residue and its corresponding function. As described in greater detail in the above Introduction, the membrane interface is a very intricate environment, delicately balanced between a polar extracellular environment and the extremely hydrophobic mass of the bilayer. Thus, in the case of the MPPs, it fits that each residue plays a specific role in membrane insertion, especially with such short residues.

*Secondary Structure of the MPPs*

While we have our hypotheses, it is not completely understood why the fluorinated mutants are more potent pore-formers than the native peptides. Another hypothesis worth testing was to determine if the fluorinated MPPs formed more highly ordered structures. It would make sense that a higher propensity to form an ordered structure may result in more effective insertion and consequent leakage. Figure 8 shows the results of a CD wavelength scan to determine the secondary structure of VAFR and
VAZR. While rather conclusive, the results do not support the hypothesis. VAFR and VAZR have nearly identical traces, displaying an ellipticity minimum at 218 nm, characteristic of a $\beta$-sheet structure. Thus, the increased leakage of VAZR compared to VAFR is not due to the fact that it has a more ordered structure, as they both display identical propensity to form $\beta$-sheets.

**Self-Assembly Before Insertion**

Another route to explore regarding the function of the MPPs is to determine if they display any self-assembly activity before insertion. Many AMPs self-assemble or oligomerize before insertion. In addition to the AMPs, leading theories insist that A$\beta$ works in a similar fashion. It is possible that the MPPs may oligomerize before inserting into the membrane, and perhaps the most effective MPPs demonstrate a higher level of self-assembly than the less potent ones.

To test the oligomerization properties, VAFR and VAZR were both subjected to a binding assay of ThT, an analog of Congo Red (Figure 10). Supporting this hypothesis, VAZR exhibited a much higher binding affinity to ThT than VAFR. Thus, in solution, VAZR forms oligomers, which may be, at least, partly responsible for the increased membrane activity. Due to its high $\beta$-sheet content and ability to bind to ThT, VAZR is only one component shy of being an amyloid, which is fibril formation. Atomic force microscopy (AFM) experiments are currently underway to determine if this series of MPPs are indeed amyloidogenic. If this is, in fact, the case, it will give provide more information toward a better understanding of amyloid toxicity.
Summary

Understanding how to design and enhance the capabilities of MPPs is of utmost importance to medical biochemistry. It is clear that fluorination has extreme potential in the rational engineering of extremely potent MPPs. The relationship between position and function needs to be better understood in order to provide a detailed comprehension of MPP activity. Further, by examining the role of self-assembly, the initial process of insertion into the membrane will be brought to light.
6. CONCLUSION

Using fluorinated phenylalanine analogs proved to be very beneficial to aiding to the overall understanding the structure—function relationship of proteins. The capability of selectively enhancing ArH···π interactions with tetrafluoro-phenylalanines is a powerful tool. These types of interactions are widely prevalent in protein structures, making $Z_{\alpha}$ and $Z_{\rho}$ very versatile components for increasing protein stability and mediating protein—protein interactions. The potential of fluorination is also applicable to enhancing the membrane activity of proteins.

Further studies must aim to decipher the intricate details of the increased MPP activity as a result of fluorination. A fuller understanding of this mechanism would elucidate the way in which peptides can form pores in a biological membrane. The ability to induce leakage from a membrane holds serious biomedical implications. By mimicking the simplistic efficacy of AMPs, the use of fluorinated amino acids could be used in the engineering of an extremely potent, novel antibiotic.

The capabilities of fluorination are remarkable. Simply mutating a select few hydrogen atoms in an entire protein system has the ability to greatly increase its stability or enhance its function. By harnessing the power of fluorination, protein engineering can be markedly improved.
7. ACKNOWLEDGEMENTS

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Sincerely,

Kris Comeforo
8. REFERENCES


