SYNTHESIS OF NEUTRAL AND CHARGED PORPHYRINS THAT SELECTIVELY BIND PHOSPHATIDYLGLYCEROL, A PHOSPHOLIPID FOUND IN BACTERIAL MEMBRANES

A Dissertation by

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Submitted to the Department of Chemistry
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Wichita State University
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

July 2014
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SYNTHESIS OF NEUTRAL AND CHARGED PORPHYRINS THAT SELECTIVELY BIND PHOSPHATIDYLGLYCEROL, A PHOSPHOLIPID FOUND IN BACTERIAL MEMBRANES

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To mom, dad, and my grandparents.
ACKNOWLEDGEMENTS

Thank you to my advisor, Dr. Dennis H. Burns, for his guidance and support over the years. I would like to express my thanks to my co-advisor Dr. William T. K. Stevenson during my first few years in the graduate program for his support and encouragement. I want to express my thanks to my committee members for their support and encouragement over the past few years. Thank you to Dr. Kevin Langenwalter for his support and help with instrumentation in order to complete my experiments. I would like to express my thanks to the WSU Chemistry department. Thank you to all my family and friends.
Picket fence porphyrins that bind specifically to phosphatidylglycerol, a bacterial membrane lipid, have been postulated as a receptor component to a synthetic antibiotic. This study reports the synthesis of neutral and charged porphyrins designed with structurally rigid binding pockets that recognize the anionic portion and the bis-hydroxyl portion of the phosphatidylglycerol (PG) anion. Structural characterizations, binding studies, and associated thermodynamics were determined using $^1$H NMR and ITC (isothermal titration calorimetry) experiments. Fluorescence correlation spectroscopy (FCS) experiments were performed to determine the selectivity and binding constants of receptor 13 in solution and synthetic vesicles containing various compositions of PG and phosphatidylserine (PS) with phosphatidylethanolamine (PE).
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LIST OF ABBREVIATIONS

AcOH  acetic acid
BF₃  boron triflouride
BOC-Gly-OH N-(tert-butoxycarbonyl)glycine
°C  degree Celsius
cal  calories
calcd  calculated
C  carbon
CD₃OD  deuterated methanol
CDCl₃  deuterated chloroform
C₆H₆  benzene
CH₂Cl₂  dichloromethane
CHCl₃  chloroform
d  doublet
D  deuterium
DMSO  dimethylsulfoxide
DMF  N, N-dimethylformamide
Et₂O  diethylether
EDAC•HCl  N-(3-Dimethylaminopropyl)-N‘-ethylicarbodiimide hydrochloride
ESI  electrospray ionization
EtOAc  ethyl acetate
F  fluorine
FCS  fluorescence correlation spectroscopy
LIST OF ABBREVIATIONS (continued)

- g: grams
- H: enthalpy
- HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt
- $\text{H}_2\text{PO}_4^-$: dihydrogen phosphate anion
- hrs: hours
- HRMS: high resolution mass spectrometry
- Hz: hertz
- ITC: isothermal titration calorimetry
- J: coupling constant
- $K_a$: association constant
- m: multiplet
- M: molar
- MeOH: methanol
- MHz: megahertz
- $\mu$L: microliters
- $\mu$S: microsiemen
- mg: milligrams
- mL: milliliters
- mol: moles
- mmol: millimoles
- MS: mass spectrometry
NH$_2$NH$_2$ hydrazine

NaCl sodium chloride

NaHCO$_3$ sodium bicarbonate

NaOH sodium hydroxide

Na$_2$SO$_4$ sodium sulfate

NH$_4$OH ammonium hydroxide

P phosphorous

Pd palladium

PE Phosphatidylethanolamine

PF$_6$ hexafluorophosphate

PG Phosphatidylglycerol

ppm parts per million

PS Phosphatidylserine

q quartet

rt room temperature

S entropy

s singlet

SnCl$_2$ stannous chloride

t triplet

TBAPG tetrabutylammoniumphosphatidylglycerol

temp temperature

THF tetrahydrofuran
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<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TFA</td>
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<td>Zn(acac)$_2$</td>
<td>zinc acetylacetonate</td>
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LIST OF SYMBOLS

\[ \Delta \quad \text{delta, or change} \]
\[ \delta \quad \text{chemical shift} \]
\[ \mu \quad \text{micro, E-6} \]
\[ \lambda \quad \text{wavelength (cm)} \]
CHAPTER 1

INTRODUCTION

1.1 General Introduction

It has been speculated that bacteria have been around for nearly 3.5 billion years and they can survive in a variety of different environments. While some of the bacteria are pathogenic, there are many species of bacteria used by industry and agriculture. For example, oil companies can use bacteria to decompose oil into carbon dioxide which reduces the environmental damage caused by the oil spill. Some bacteria that are found in soil can convert inorganic gases into various forms of carbon and nitrogen necessary for crop production [1].

The topic of antimicrobial or drug resistance is not new, but the number of organisms becoming resistant is growing at extraordinary rates [2]. Infectious diseases, caused mostly by bacteria, became one of the top five causes of death in the United States by 1995 [3].

Multidrug resistant bacteria are becoming a problem for worldwide health. With the introduction of antibiotics or “miracle drugs”, it was thought that diseases and infections would disappear [3, 4]. Within the past 50 years, resistance to all new antibiotics has been observed [5]. Improving the properties of current antibiotics has been explored, but the modified antibiotics follow the same mechanism of attack as the previous drugs [6].

By the year 2020, the Infectious Diseases Society of America has called for the development of ten new classes of antibiotic drugs in response to the growing number of antibiotic resistant bacteria [7]. *Staphylococcus aureus* bacterial strains acquired in the hospital and communities exhibited resistance to penicillin approximately 20 years after penicillin was
introduced as a treatment for infections in the early 1940s [8], and in the late 1990s signs of resistance to vancomycin began to emerge [9]. The well-known strain MRSA (methicillin-resistant *S. aureus*) was discovered in the 1960s shortly after the introduction of methicillin. Vancomycin has been used to treat MRSA because of its resistance to most antibiotics, but the emergence of vancomycin-resistant *S. aureus* (VRSA) has begun [1].

In 2009 it was reported by Rekha Bisht et al. that approximately 70% of infections caused by bacteria in the hospitals are resistant to at least one antibiotic [10]. Since some of the bacteria have become resistant to approved antibiotics, doctors have to prescribe experimental antibiotics that could be potentially harmful or re-use old antibiotics that have poor safety profiles, such as colistin [10, 11]. Colistin is very effective against gram-negative bacteria and was used in Japan and Europe in the 1950s. Neurotoxicity, renal toxicity, and nephrotoxicity were most harmful effects that were reported from treatments using colistin. The adverse effects of renal toxicity and neurotoxicity were dose-dependent, and the effects could be reversed upon completion or discontinuing the treatment. However, it was documented in a few reports that the effects of nephrotoxicity were not reversible [12]. Today, treatments with colistin have been restricted to patients with cystic fibrosis.

It has been documented that Staphylococci and Pneumococci (*Streptococcus pneumonia*) have shown an increase in resistance to antibiotics. Approximately 25% of bacterial pneumonia cases are now showing signs of resistance to Penicillin. However, more cases of bacterial pneumonia (~25%) are exhibiting signs of multidrug resistance [10].
Due to the rapid emergence of drug resistant bacteria, new antibiotics have been discovered and newer ones are still emerging.

Some of the recent new antibiotics have scaffolds that have not been used in prior antibiotics. Other antibiotics have had their scaffolds modified, such as oxazolidinones (Figure 2). DuPont discovered the oxazolidinone scaffold and pursued the study of DuP-721, but the study was cancelled due to the compound’s toxicity. Modification of the scaffold led to the synthesis of linezolid which is active against MRSA, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococcus faecium*. Linezolid is currently on the market as the antibiotic Zyvox [7, 13].
Bacteria can exchange genetic information and thereby acquire new resistance genes which can be found on chromosomal DNA and plasmids. The plasmids can be transferred from one bacterium to another and will transfer the resistance genes to other bacteria. The transfer of genetic information occurs more through the transfer of plasmids than gaining new DNA [14]. In 2011, Europe experienced a hemolytic uremic syndrome outbreak that was caused by an *E. coli* strain that had become resistant to β-lactam antibiotics after obtaining a plasmid [7] that encoded the CTX-M-15 enzyme [15], a CTX-M variant that is an extended-spectrum β-lactamase.

Bacteria have four principal ways to resist antibiotics, and the resistance can occur via one or more of the four pathways [6]. These mechanisms (Figure 3) of antibiotic resistance are enzymatic inactivation, reduction in uptake, removal of antibiotics, and target site alteration [14]. The mechanisms of resistance are shown in the diagram below.
The decline in the effectiveness of the current antibiotics has led to a search for new therapeutics. Research focus has included antimicrobial peptides, which are a part of the innate immune system, as a potential solution [5] because of their wide range of activity against many different types of bacteria and fungi [16, 17]. There are now several hundred peptides that have been identified as a result of the ongoing research.
1.2 General Overview of Antimicrobial Peptides

Antimicrobial peptides have four structural classes, but the α-helical and β-sheet structures are more predominant in nature than the other two structures. The peptides, containing 12 – 50 amino acids, have an overall positive charge [18] and have an amphiphilic character (Figure 4) [20].

![Amphiphilic structure of an antimicrobial peptide](image)

Figure 4. Amphiphilic structure of an antimicrobial peptide [21].

The focus on antimicrobial peptides as a possible therapeutic originated from problems caused by bacteria being resistant to current antibiotics. Advantages to using antimicrobial peptides include a lower probability of resistance [19] and rapid onset of activity [22]. Despite having a broad-spectrum of activity, antimicrobial peptides have not been approved by the FDA [22].

The antimicrobial peptide’s cationic property allows them to have favorable Coulombic interactions with bacterial cells due to the negative charge found on the cellular membrane. The membrane on a bacterial cell has a greater negative charge than mammalian cells which allows
the antimicrobial peptides to have cell selectivity [16]. Bacteria have anionic phospholipids located on the outer leaflet of the inner membrane whereas eukaryotic cells contain mostly zwitterionic lipids in the outer leaflet.

Antimicrobial peptides commonly act as membrane-disrupting agents of a bacterial cell [17, 23] which occurs via the nonpore carpet or the pore forming barrel-stave and toroidal mechanisms (Figure 5). It is proposed that the peptides initially carpet the membrane before integration into the membranes [21]. Upon integration into the membranes the hydrophobic region of the peptide faces the membrane as it inserts, while the hydrophilic region faces the water-filled pore [24].

Figure 5. Illustration of the three models that show antimicrobial peptides interacting with the cellular membrane. The peptides can interact in one of the possible methods: barrel-stave (a), toroidal pore (b), and carpet (c) [19].
The toroidal pore and the barrel-stave models for pore formation are similar to one another. The difference between the two models is the linking of the antimicrobial peptide to the inner and outer lipid bilayers. Unlike the pore formation in the barrel-stave and toroidal models, the carpet model has antimicrobial peptide molecules that surround a fragment of the cell membrane. According to Lazarev and Govorun, the toroidal pore model is the model that most antimicrobial peptides follow [19]. There have been other models that propose various mechanisms for the actions of antimicrobial peptides. Even with so many models, the exact mechanisms for all of the various models are still unknown [19].

There are problems associated with using the peptides as therapeutics, such as exhibiting host toxicity [25]. Other problems include cost of preparation and serum proteolytic degradation [26]. Serum salts that are present may alter the structure of the peptide which will change the behavior of the peptide [27]. To overcome these problems, researchers are making antimicrobial peptide mimics [28].

1.3 General Overview of Phospholipids

The differences in composition (Figure 6) highlight the basis as to why antimicrobial peptides are selective towards prokaryotic cells (bacterial cells). Eukaryotic cells have a high concentration of zwitterionic phospholipids, lipids with no net charge, on the outer leaflet of their cellular membrane. Negatively charged phospholipids are located in the inner leaflet of a eukaryotic cell, but prokaryotic cells have a higher concentration of anionic phospholipids on the outer leaflet [29, 30].
Figure 6. Cationic peptides favor interactions with anionic phospholipids due to electrostatic interactions. Bacterial cell membranes have high concentrations of anionic phospholipids [29].

Phospholipids have amphipathic properties and are comprised of three different regions. The three regions are: a polar head group, an interfacial region that is of intermediate polarity, and a hydrophobic tail [31]. Figure 7 contains a common structural formula for glycerophospholipids which are defined by an esterification of a phosphate group on one of the oxygens of the glycerol molecule [32].
The $R_1$ and $R_2$ can be saturated or unsaturated alkyl groups, hydroxyl, or cyclo fatty acids [33]. The symbols $R_1$ and $R_2$ represent long-chained carboxylic acids that are linked to glycerol at the primary and secondary alcohol positions [34].
Among the phospholipids present in nature, the most studied are the phosphatidylcholines (PC), phosphatidylethanolamines (PE), and phosphatidylglycerol (PG). The PE and PG lipids are responsible for various functions. The PE lipids aid in processes such as the formation of bilayer curvature, cell division, and fusion. PG forms strong electrostatic interactions with other charged lipids. These interactions allow the lipids to act as stabilizers [35].

PEs are zwitterionic phospholipids that are found in both eukaryotic and prokaryotic cells. PGs make up a large portion of a bacterial membrane. While the normal percentage for PG in a bacterial membrane is around 25%, some bacteria have much higher PG percentages. For example, the *Staphylococcus aureus* contains up to 80% PG while other bacteria have lower PG percentages [35].

In prokaryotic cells PG and PE are the major lipid components. For example, *Escherichia coli* contain 70-80% PE and 20-25% PG [36]. In contrast, eukaryotic cells have much lower concentration of the PE phospholipid in comparison to the other membrane lipids and do not contain PG. As seen in Figure 8, the lipid distribution in eukaryotic and prokaryotic cells is an asymmetric distribution between the inner and outer leaflets.
Figure 8. Lipid distribution across the inner and outer membrane of human erythrocyte and *Bacillus megaterium* cells [37].

*Bacillus megaterium*, a gram positive bacteria, is composed of 30% PG and 70% PE, but most of the PE exists in the inner membrane and PG only exists in the outer membrane [37]. In comparison to *Bacillus megaterium*, the erythrocyte contains PE in a smaller percentage and contains no PG.
In this report, we describe the synthesis of receptors that will bind to phosphatidylglycerol which is found in the bacterial membrane. The goal of this research was the design of neutral and charged porphyrins that would provide a complementary binding pocket for the head group of the PG anion as depicted in Figure 9. The structure of the receptor
allows for the proper alignment of the urea and ammonium pickets with the multifunctional lipid head group needed for binding. These types of receptors are intended to make conjugates with antimicrobial mimics to increase their selectivity for prokaryotic cells for therapeutic purposes.
CHAPTER 2

SYNTHESIS OF RECEPTORS

2.1 Synthesis of the Porphyrin Base

The synthesis of the neutral and charged receptors all followed the same initial procedures when preparing the porphyrin scaffold. The synthesis of the receptors began with the preparation of tetra-nitro porphyrin 1. The crude tetra-nitro porphyrin was purified by column chromatography to remove any polymeric by-products. The nitro groups were reduced to the amino group using SnCl$_2$•2H$_2$O in concentrated HCl [38]. When following the procedures above set forth by Collman, four different porphyrin isomers were synthesized. The tetra-amino porphyrin 2 was isomerized to furnish the tetra-alpha atropisomer following a method developed by Lindsey.

![Figure 10. Four possible porphyrin atropisomers [39]](image)

The $\alpha,\alpha,\alpha,\alpha$-atropisomer makes up ~1/8 of the overall yield of the four isomers. By adding the meso-tetrakis(o-aminophenyl)porphyrin to silica gel in benzene, the yields of the
α,α,α,α-atropisomer increased dramatically. Of the various atropisomers, the α,α,α,α-atropisomer has the highest affinity for silica gel [40]. The alignment of the four amino groups in the same direction is due to the intermolecular interactions with silica gel.

A green band was observed on the column when purifying the α,α,α,α-atropisomer. A solvent system comprised of CHCl₃:Et₂O (1:1) was used to elute the green band [38]. After the elution of the first impurity, the solvent was switched to C₆H₆:Et₂O (1:1) to remove the three undesired atropisomers. However, due to the high cost of benzene, toluene was used instead. After the eluants became pale in color, the α,α,α,α-atropisomer was removed when the solvent was switched to acetone:ether (1:1) [38, 40].

Following the procedure set forth by Lindsey, TLC was used to carefully monitor the progress of compound 3 as it was being eluted off the column. By following the progress of the α,α,α,α-atropisomer with TLC, it was noted that the unwanted atropisomers were still present on the column. These atropisomers were also observed in the NMR spectrum of the α,α,α,α-atropisomer product.

By following the procedure designed by Lindsey, some of the unwanted atropisomers were found to be in the fractions containing the α,α,α,α-atropisomer product. Therefore a second purification method was created to purify the α,α,α,α-atropisomer. Using a sintered glass funnel with vacuum filtration, the benzene slurry was evaporated to dryness. To the dried silica powder, was added a CHCl₃:Et₂O (1:1) in order to remove the green band. The solvent was changed to toluene:Et₂O (1:1) to elute off the three undesired atropisomers and then changed to 3.75% isopropylamine in diethyl ether to yield the desired α,α,α,α-atropisomer. By following this procedure, the ¹H NMR of 3 did not show any of the other three atropisomers byproducts.
The TLC of the desired α,α,α,α-atropisomer fraction, showed only one spot on the TLC plate, unlike the previous method that used column chromatography designed by Lindsey.

Scheme 1. Synthesis of compound 3.

2.2 Synthesis of the Neutral Porphyrin Receptors

When designing the neutral porphyrins, various pickets were chosen to create binding pockets of various sizes that would bind the head group of the PG lipid. The pickets on the porphyrins were designed to align the urea pickets with the oxygens from the glycerol and phosphate portion of the head group from the PG anion. The urea functionality on the porphyrins allowed for hydrogen bonding with the oxygens on the head group of the lipid.
The pickets on the porphyrins provided additional stabilizing forces to the PG lipid. Compounds 7 and 10 contained pickets that would provide hydrophobic interactions. The hydroxy pickets in compound 9 provided hydrophilic interactions. Pickets in compound 8 added additional stabilizing forces through hydrophobic and dipole-dipole interactions [41].

2-\textit{O}-Benzylethanolamine provided a picket that was large with a bulky benzyl group at the end of the picket. The synthesis of 2-\textit{O}-Benzylethanolamine was performed by Champika Jayasinghe according to the procedure developed by Kolomiets, et al.

Figure 11. Neutral receptors synthesized.
Triethyleneglycol monoethylether was used to synthesize the second large picket. The use of compound 6 as the picket provided dipole-dipole interactions instead of using the bulky benzyl group. Scherman, et al. developed a procedure to convert triethyleneglycol monomethylether to the corresponding amine which was used to synthesize compound 6.

Scheme 3. Synthesis of compounds 5 and 6 [43].

With the Gabriel synthesis, the reaction between 5 and potassium phthalimide produced the corresponding phthalimide glycol. Deprotection was carried out using hydrazine monohydrate, and 6 was isolated in a 62% yield [43].
The preparation of the urea pickets followed two general synthetic routes. In one route, the first step involves the formation of an isocyanate intermediate from the phenyl amine using phosgene or a phosgene derivative and then the formation of the urea. The second route is the reaction that occurs between the phenyl amine and a reagent containing an isocyanate group.

Scheme 4. General reaction scheme of an amine with a phosgene derivative.

Scheme 4 illustrates the general synthetic pathway that was followed for the attachment of the pickets to the free base porphyrin. With the toxicity concerns of phosgene gas, various derivatives have been used in the synthesis of ureas. One such derivative is triphosgene, a solid
and easily handled, which can be used in one of the possible synthetic pathways for synthesis of urea compounds [44].

Scheme 5. General synthetic scheme for synthesis of urea receptors.

The synthetic route for most of the porphyrin receptors began with addition of triphosgene to the $\alpha,\alpha,\alpha,\alpha$-atropisomer. The amino groups on the $\alpha,\alpha,\alpha,\alpha$-atropisomer were converted to four isocyanate groups to yield the reaction intermediate. The urea functionality was furnished after the addition of the amine to the reaction intermediate.

According to Collman and co-workers the isocyanate intermediate is highly moisture sensitive; therefore, the conversion of the anilines to the ureas should be done as a one-pot reaction [45]. To prevent water from generating the carbamic acids, the reaction was carried out in a glove box.
The synthesis of compounds 7 and 8 was developed according to procedures designed by Collman and co-workers. Compound 3 was dissolved in methylene chloride and triethylamine at room temperature. Triphosgene (1.5 equivalents) was added to the reaction mixture to generate the isocyanate precursor. The corresponding amine was added to the isocyanate to create the desired urea.

The percent yields of receptors 7 and 8 were 18% and 30%, respectively.

In an attempt to improve porphyrin 7 yields compound 4 was reacted with triphosgene to furnish the isocyanate. The $^1$H NMR of the crude material furnished from the synthesized isocyanate and porphyrin 3 did not show an improvement in the yield of compound 7. Therefore, this pathway was not continued.

Cleavage of the benzyl groups from 7 was attempted by hydrogenation using Pd/C in a Parr apparatus. The $^1$H NMR of the compound 7 exhibited sharp distinct resonances for the aromatic hydrogens. After the hydrogenation, the $^1$H NMR of the crude product showed many complex resonances. The complexity of the resonances is speculated to be caused by possible over reduction of 7.

Due to the possible over reduction, the addition of compound 7 to ethanethiol and boron trifluoride etherate provided an alternative pathway for synthesizing 9 [46]. The $^1$H NMR of the crude sample showed much sharper resonances unlike the hydrogenation of 7 in the presence of palladium on carbon. Following this pathway, the crude $^1$H NMR showed the appearance of a broad resonance at 4.35 ppm, indicating the formation of hydroxyl groups from the cleavage of the benzyl groups.
The resulting crude product was purified using radial chromatography. Due to polarity of the molecule, a ternary solvent mixture of CH$_2$Cl$_2$:methanol:H$_2$O was required to elute the molecule from the silica gel. The desired product was isolated in 25% yield. Prep thin-layer chromatography was explored as a possible method for purification of 9; however, the $^1$H NMR of the isolated material showed the disappearance of the two hydrogens attached to the nitrogens of the pyrrole rings. The disappearance of these two hydrogens is indicative of metalation. Several methods were attempted to demetalate the porphyrin, but none of the attempts were successful in furnishing the desired product.

The reaction of isocyanates with amines can also be utilized to create various ureas. The addition of propyl isocyanate to 3 was used to furnish 10. The receptor was isolated in 20% yield.
The addition of zinc was used to enhance the rigidity of porphyrin 10. It was speculated that the addition of the metal could also provide an additional point of binding [47].

To metallate 10 the porphyrin was treated with 3 equivalents of zinc acetylacetonate (Zn(acac)$_2$). The reaction was allowed to proceed for 5 hours at room temperature, followed by removing the solvent under reduced pressure. The resulting material was triturated with diethyl ether until there was no color observed. The Zn(II) metallated porphyrin was collected in 75% yield [48].
2.3 Synthesis of the Charged Porphyrin Receptors

The synthesis of compound 12 (scheme 10) started with the α,α,α,α-atropisomer 3. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDAC) was added to a solution that contained α,α,α,α-atropisomer and BOC-glycine in an ice bath. The reaction was allowed to stir overnight at 0°C in a dewar to yield the crude product which was isolated by column chromatography. Compound 12 was isolated with a 62% yield [49].
Scheme 10. Synthetic scheme of compound 12.

EDAC alone proved to be the best reagent to use when coupling 3 with the BOC protected glycine. Coupling 3 and BOC-glycine was first attempted in the presence of trimethyl phosphite and iodine, but use of this reagent did not furnish compound 12. It is speculated that the formation of methyl iodide could be preventing the amidation from occurring. Trimethyl phosphite may be reacting with the byproduct, methyl iodide, via the Arbuzov reaction instead of forming the active intermediate.
Scheme 11. Formation of alkyl phosphonate via Arbuzov reaction.

Using hexafluorophosphate counterions with the charged ammonium groups was expected to increase the solubility of the receptor in organic solvents and allow for the formation of X-ray quality crystals. By cleaving the BOC groups with HCl, the hexafluorophosphate anion could then be exchanged for the chloride ions. The first attempt at cleavage of the BOC groups and formation of the ammonium ions was attempted using a 0.5 M HCl in methanol. After evaporation of the solvent, it was evident that the HCl had no effect on the BOC groups.

Hexafluorophosphoric acid was used to in an attempt to cleave the BOC groups since the previous reactions conditions failed. Cleaving the BOC groups using hexafluorophosphoric acid, would provide a direct approach to the addition of the hexafluorophosphate ions and would not require an anion exchange in order to produce the desired receptor. As with the previous reactions that used HCl, the addition of hexafluorophosphoric acid did not produce the desired receptor.
Deprotection of the BOC groups was accomplished using TFA in methylene chloride at 0°C. After evaporation of the solvent mixture, the crude solid was triturated with diethyl ether to remove excess TFA. Under the acidic conditions the ammonium functionality was created with trifluoroacetate counterions which could be exchanged for hexafluorophosphate ions. Compound 13 was isolated in 62% yield, but the yield was not optimized.

The final charged receptor was synthesized by the complete exchange of the trifluoroacetate anion with the hexafluorophosphate anion. To compound 13 was added 20 equivalents of ammonium hexafluorophosphate at room temperature. The reaction was allowed to stir for 24 hours followed by a water wash.

The fluorine NMR showed two fluorine resonances in the spectrum at -70.17 ppm and -74.32 ppm, which indicated the incomplete anion exchange. For a pure product, the $^{19}$F NMR should show a doublet due to the coupling between the fluorine and phosphorus atoms. If there
had been no conversion, the $^{19}$F NMR of the starting material would have shown a singlet as the only resonance at -74.32 ppm.

To complete exchange of the trifluoroacetate anions, another 20 equivalents of ammonium hexafluorophosphate was added to the redissolved crude compound in ethyl acetate. After the reaction mixture was washed with water, the $^{19}$F NMR showed a single resonance split as a doublet at -70.17 ppm and the $^{31}$P NMR showed a septet at 107.945 ppm. The yield of compound 14 was not optimized, but it was isolated in 51% yield.

Two additions of ammonium hexafluorophosphate were needed to overcome the reaction equilibrium and to complete the anion exchange. Ammonium hexafluorophosphate (20 equivalents) was added to Compound 13 in ethyl acetate. The reaction was monitored by $^{19}$F NMR after 24, 48, and 72 hours. Each of the three fractions showed an incomplete conversion by the observation of two resonances that resulted from the hexafluorophosphate anions and the trifluoroacetate anions.

Another 20 equivalents of ammonium hexafluorophosphate was added to each of the three fractions and allowed to stir for 48 hours. A porphyrin by-product increased in yield by allowing the first addition of ammonium hexafluorophosphate to proceed for 48 and 72 hours. The by-product was observed in the $^1$H NMR after the second addition of ammonium hexafluorophosphate. Allowing the first addition of ammonium hexafluorophosphate to react for 24 hours, no porphyrin by-product was observed in $^1$H NMR after the second addition of ammonium hexafluorophosphate. After the second addition of ammonium hexafluorophosphate, the $^{19}$F NMR showed a doublet resulting from the hexafluorophosphate ions.
Scheme 13. Synthetic Scheme of compound 14.
CHAPTER 3

ANION BINDING STUDIES

Anion binding studies used NMR and ITC to determine the associated binding constants and associated thermodynamics of receptor-anion complexation. Receptor-anion binding stoichiometry was determined by Job plots using $^1$H NMR. After determination of the binding stoichiometry, the binding constant of the receptors was determined. The binding constants were determined by non-linear regression analysis using EQNMR of the isotherms afforded by $^1$H NMR titration studies [50]. See experimental for details.

3.1 NMR Characterizations of the PG Anion

Obtaining crystal structures of the receptor-anion complex has proven to be extremely difficult, thus the need for characterization of the PG anion. The advantage of the PG anion in comparison to the $\text{H}_2\text{PO}_4^-$ anion is being able to monitor the chemical shift changes of the protons on the head group during complexation. With the use of various NMR experiments, insight into the binding motif of the receptor-PG complex could be obtained. In order to gain insight on the complexation, different NMR experiments were used to characterize the PG anion in DMF-d$_7$. COSY was used to determine which protons were coupled together. HMQC and HMSQC were used to determine which protons were coupled to which carbons. DEPT was used to corroborate the proton assignments made from the COSY, HMSQC, and HMQC NMR experiments.
The protons (6 and 7) attached to the carbon ester reside at 4.4 and 4.2 ppm in the form of a doublet and multiplet. Proton 5 on the carbon ester is a multiplet at 5.2 ppm. Each of the proton environments integrates to one proton. The proton (2) on the methine carbon originates as a multiplet at 3.55 ppm and is partially buried from overlap of the hydrogens from the tetrabutylammonium counter ion. The methylene protons (1) reside at 3.45 ppm. The methylene protons (3 and 4) that form the phosphate ester are located at 3.9 ppm.

Figure 12. $^1$H NMR characterization of protons in head group of PG anion.

Figure 13 is an expansion of the protons that are associated with the head group of the PG anion. The COSY shows that the methine proton 5 is coupled to the methylene proton 6. The methine proton 5 is coupled to proton 6, but is not coupled to proton 7. Since no coupling can be observed on COSY between proton 5 and proton 7, this suggests that the dihedral angle is $90^\circ$ between these two protons. This is consistent with a structure that has the two hydrophobic chains parallel to one another and an orthogonal head group. The COSY NMR shows proton 6
being coupled to proton 5 and proton 7. Proton 7 is geminal coupled to proton 6 and exists as a doublet of doublets in $^1$H NMR due to the existence of diastereoisomers.

Figure 13. COSY NMR of the PG anion head group
HMQC and HMSQC were used to verify the proton assignments that were made from the COSY and $^1$H NMR. Both the HMQC and HMSQC showed correlation between proton 5 and the methine carbon that resonates at 71 ppm. Protons 6 and 7 are coupled to a methylene carbon resonating at 63 ppm. Proton 2 that resonates at 3.55 ppm is coupled to the methine carbon that resonates at 73 ppm.

Figure 14. HMQC (top) and HMSQC (bottom) NMR of the head group of PG anion.
The DEPT experiment of PG anion can be used as further verification of the PG head group proton and carbon assignments. In the DEPT experiment, all methylene carbons are pointing downwards. All methyl and methine carbons point upwards. In the PG molecule there are two types of methyl carbons and two methine carbons, indicating there should be four carbon environments pointing up.

![Figure 15. DEPT NMR experiment of PG anion.](image)

In the DEPT NMR experiment, the two resonances that are near 12 ppm correspond to the two types of methyl carbons at the end of the lipid hydrophobic tail and the tetrabutylammonium counter ion. The two resonances at 71 and 73 ppm correlate to the two
methine carbons 2 and 5. In the HMQC and HMSQC the two methine resonances correlate to the proton resonances at 5.2 and 3.55 ppm.

With the $^1$H NMR inverse titration, compound 8 was titrated into a PG solution. The proton environments of the PG were monitored throughout the addition of 8.

![Figure 16](image.png)

Figure 16. Inverse $^1$H NMR titration with 0.0, 0.2, 0.4, 0.8, and 1.25 equivalents of compound 8 added to PG.

By monitoring the chemical shifts, it is possible to observe how the addition of 8 affects the environment of the PG anion protons. The proton (2) on the methine carbon in the glycerol head group had a much larger upfield chemical shift than the methine proton 5. With the chemical shift of proton 2 being much larger than the chemical shift of proton 5, this indicates the proton is positioned above the porphyrin ring and is highly exposed to the ring current of the porphyrin
scaffold. The chemical shift of the methine proton 5 was influenced by the porphyrin scaffold’s ring current, but not to the extent as proton 2. This experiment suggests that the head group of the PG anion is inside the binding pocket of the receptor and lies above the plane of the ring. Since proton 2 was influenced greatly by the ring current of the porphyrin scaffold, it must be positioned deeper in the binding pocket than proton 5 and close to the porphyrin scaffold [41].

3.2 $^1$H NMR Experiments of Neutral Receptors

Dihydrogen phosphate was used to correlate the binding of the receptor with the phosphate anionic portion of PG. The Job plot was determined by $^1$H NMR using the dihydrogen phosphate anion and porphyrin 7. The binding stoichiometry for the receptor was 1:2 which indicates two PG anions would bind to one receptor molecule.
Figure 17. Job plot of compound 7-H₂PO₄⁻ complex in DMF-d₇ at 30°C.

The binding stoichiometry of 7 with dihydrogen phosphate is the same as other previously recorded tetra urea pickets with dihydrogen phosphate that have been examined in the past [51,52]. The crystal structure of a tetra urea α,α,α,α-atropisomer receptor with two dihydrogen phosphate anions can be seen in Figure 18.
Figure 18. Crystal structure of α,α,α,α,5,10,15,20-tetrakis(2-(4-fluorophenylurea)phenyl porphyrin complex with two dihydrogen phosphate anions [52].

The dihydrogen phosphate anions are oriented in a way that promotes hydrogen bonding between the pickets and the anions. The oxygen atoms of the anion are bound to two of the urea pickets via hydrogen bonding [52].

The number of PG anions bound to compounds 7 and 8 was found to be different than dihydrogen phosphate. As determined from the Job plot, the binding stoichiometry for the PG anion was 1:1 for both receptors.
Figure 19. Job plot of compound 8-PG complex in DMF-d$_7$ at 30°C.
NMR isotherms were obtained for 7 and 8 by monitoring the chemical shifts of various protons on the receptor when titrated with PG in DMF-d$_7$. Binding isotherms for porphyrins 7 and 8 were generated by following the chemical shift changes of protons 1, 2, and 3 (Figure 21). The binding constants were calculated from the $^1$H NMR isotherms utilizing EQNMR nonlinear regression analysis. The binding constants for 8 and 7, averaged from 3 experiments, are 6500 M$^{-1}$ (9% error) and 2400 M$^{-1}$ (13% error), respectively.
Figure 21. Chemical shifts of the labeled protons from porphyrins 7 and 8 that were monitored during $^1$H NMR titration.

Figure 22. $^1$H NMR titration curve of compound 7 at 30°C in DMF-d$_7$. 
It was predicted that the porphyrin 9 would form 1:1 binding with the PG anion. The pickets each had a hydroxyl group that was hypothesized to form hydrogen bonds with each other, therefore creating a defined and structurally rigid binding pocket for the head group of the anion. Porphyrin 9 did not show 1:1 binding with the PG anion.
Figure 24. Job plot of compound 9-PG complex in DMF-d$_7$ at 30°C.

Upon heating to 40-50°C, the receptor formed multiple atropisomers due to unstable pickets. Compound 10 also showed the formation of atropisomers at the same temperatures as 9. Compounds 7 and 8 were stable up to 100°C and did not show multiple atropisomers. With no formation of atropisomers, 7 and 8 had created a defined binding pocket that was structurally rigid [41].

Compound 10 behaved similarly to 9. The binding stoichiometry was a mixture of 1:1 and 2:1 according to the Job plot. The multiple binding stoichiometries are thought to be caused
by the flexibility of the pickets on the porphyrin, which causes poor alignment of functional
groups between the receptor and PG in the receptors binding pocket.

![Figure 25. Job plot of compound 10-PG complex in DMF-d$_7$ at 30°C.](image)

The addition of Zn(II) did not change the binding stoichiometry of porphyrin 10. The Job
plot of porphyrin 10 indicated the complex binding stoichiometry. It was believed that Zn(II)
would increase the rigidity of the porphyrin and provide an additional binding site for the anion
which would lead to 1:1 binding stoichiometry. The increased rigidity of the porphyrin may
have affected the urea pickets from being able to align properly with the anion [47].
3.3 ITC Characterizations of the Neutral Receptors

Compounds 7 and 8 were the only two neutral receptors to show 1:1 binding stoichiometry with the PG anion. After determination of the binding constants through NMR titrations, the two receptors were subjected to isothermal titration calorimetry (ITC). ITC studies allowed for determination of the thermodynamic properties of the binding process between the PG anion and receptor in solution. All ITC studies were conducted at 30°C in a CHCl₃:DMF (5:95) solvent mixture.
The ITC titration curve for 7 is an exotherm.

Figure 27. ITC exotherm of compound 7 in a DMF solution containing 5% CHCl₃ at 30°C.

The binding constant was 2100 M⁻¹ (5% error). The measured ΔH was -2830 cal/mole (3% error) and the ΔS was calculated to be 6 cal/mol/°C. Compound 8 produced an exotherm when subjected to ITC. The binding constant was determined to be 3700 M⁻¹ (12% error). The determined ΔH was -1210 cal/mole (3% error) and ΔS was calculated to be 12 cal/mol/°C.
Figure 28. ITC exotherm of compound 8 in a DMF solution containing 5% CHCl₃ at 30°C.

The ITC and NMR binding constants for each receptor were close in value to each other. In both experiments, the binding constant was higher for 8. ITC experiments for both compounds produced negative ΔH values and positive ΔS values. These results show that the binding of the PG anion to the receptor is both enthalpy and entropy driven. The negative enthalpies result from the hydrogen bonding in the receptor-anion complex [41, 53]. Receptor 8
had a higher ΔS value than receptor 7, which could be attributed to the release of more solvent molecules upon complexation to the lipid due to the larger picket [41, 53]. The negative ΔH values and positive ΔS values indicate that the entire picket is involved in the binding of the lipid to the receptors.

3.4 NMR and ITC Characterizations of the Charged Receptor

The binding stoichiometry of 14 was 1:1 with the PG anion. The binding constant was calculated to be 4900 M$^{-1}$ (11% error) by averaging the results from two experiments. In order to obtain repeatable results and sharp resonances, the $^1$H NMR titrations were conducted in 40% DMSO-d$_6$ in CDCl$_3$. Figure 31 shows the protons that were followed throughout the $^1$H NMR titration.

![Graph showing Job plot](image)

Figure 29. Job plot of compound 14-PG complex in a solution of 40% DMSO-d$_6$ in CDCl$_3$ at 30°C.
Figure 30. $^1$H NMR titration of compound 14-PG complex in a solution of 40% DMSO-d$_6$ in CDCl$_3$ at 30°C.

![Graph showing chemical shifts](image)

Figure 31. Chemical shifts of the labeled protons from porphyrin 14 that were monitored during $^1$H NMR titration.
With crystal structures of the complex being difficult to produce, an inverse titration was used to provide insight into the binding motif. By examination of the stacked $^1$H NMRs in Figure 32, the protons on the head group of the PG anion are observed to be moving upfield indicating the PG anion is located above the porphyrin scaffold within the binding pocket.

**Figure 32.** Inverse $^1$H NMR titration of compound 14 with PG in CDCl$_3$ with 40% DMSO-d$_6$. 0.0, 0.05, 0.10, 0.15, 0.2, 0.4, and 0.6 equivalences of porphyrin 14 added.

Porphyrin 14 was titrated with dihydrogen phosphate and the PG anions in CDCl$_3$ with 40% DMSO-d$_6$. The stacked plots (Figures 33 and 34) show the ammonium protons (7.9 ppm)
moving downfield upon titration with both anions and indicating binding to the ammonium protons via hydrogen bonding. The methylene protons (2.8 ppm) move upfield in both Figures due to diamagnetic shielding. The amide protons (9.0 ppm) move downfield upon titration with PG, but move upfield upon titration with dihydrogen phosphate. Prior to the addition of dihydrogen phosphate and PG, DMSO was located with the pocket of the porphyrin acting as a hydrogen bond acceptor. Upon addition of the PG anion, DMSO would be expelled from the binding pocket allowing the PG anion to insert the head group into the binding pocket to form hydrogen bonds with the amide protons. The upfield chemical shift of the amide protons upon titration with dihydrogen phosphate suggests the protons are no longer forming hydrogen bonds.

![Figure 3. 1H NMR titration of Compound 14 with 0, 0.6, 1.0, and 2.5 equivalents of dihydrogen phosphate](image)

Figure 33. 1H NMR titration of Compound 14 with 0, 0.6, 1.0, and 2.5 equivalents of dihydrogen phosphate
Figure 34. $^1$H NMR titration of Compound 14 with 0, 0.6, 1.0, and 2.5 equivalents of PG.

The ITC of 14 was difficult to obtain in 40% DMSO in CHCl$_3$. There would be a second endotherm that would form at the end of the experiment from possible aggregation in the mixture or there was a second type of reaction. To overcome potential aggregation and to obtain repeatable results, the solvent mixture was changed to DMSO:CHCl$_3$:MeOH (5:4.5:0.5) at 40°C. The $\Delta$H and binding constant that were obtained for the formation of the receptor-anion complex was 691 cal/mol (13% error) and 2800 M$^{-1}$ (36% error). The $\Delta$S was 18.0 cal/mol/°C.
Figure 35. ITC endotherm of compound 14 in a DMSO:CHCl₃:MeOH (5:4.5:0.5) solution 40°C.
CHAPTER 4

FLUORESCENCE CORRELATION SPECTROSCOPY STUDIES

4.1 Fluorescence Correlation Spectroscopy (FCS)

FCS was used to determine the binding affinity and selectivity of porphyrin 13 to synthetic vesicles containing PG. The data presented in this chapter was collected in collaboration with Zifan Wang and Dr. English. My role in this study was the preparation of porphyrin 13 and the lipid vesicles.

The following discussion provides a brief introduction into FCS and the data obtained using FCS. Fluorescence correlation spectroscopy can be used to analyze the fluorescent intensity fluctuations from one molecule or several molecules diffusing through a small observation volume defined by a focused laser [54].

Intersystem crossing and diffusion are the primary processes that can cause the intensity fluctuations. Diffusion coefficients can be obtained directly by analysis of the autocorrelation decay. Binding affinities can be obtained by observing the changes in the diffusion coefficient of a fluorophore that has bound to another molecule or particle. In this study the PG receptor, porphyrin 13, was the fluorophore and changes in its apparent diffusion constant occurred when it was bound to vesicles containing PG. Because the vesicle is approximately 100 times larger in diameter than the porphyrin, a decrease in the observed diffusion coefficient was easily measured. In addition, because the porphyrin has a high triplet yield there are substantial fluctuations due to intersystem crossing and triplet state decay and this effect was also included in the analysis.
In the FCS experiments the fluorescence emission intensity is proportional to the number of particles present in the observation volume, and changes in the number of molecules in the small observation volume results in fluorescent intensity fluctuations [55]. Figure 36 depicts data from a fluctuating signal. The fluctuations, δF(t), in the fluorescence intensities are defined (equation 4.1) by deviations from the time-based average fluorescent signal, <F(t)>, and the instantaneous fluorescent signal, F(t) [56, 57].

\[
\delta F(t) = F(t) - \langle F(t) \rangle
\]  

(4.1)

![Figure 36. Depiction of fluorescent signal fluctuations [58].](image)

FCS data is treated by performing autocorrelation analysis of the time dependent fluorescence signal. The autocorrelation function is given in equation (4.2).

\[
G(\tau) = \frac{1}{T} \int_0^T \delta F(t) \cdot \delta F(t + \tau) dt = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}
\]  

(4.2)

The function compares the fluctuations of fluorescence intensity at time t with the fluorescence intensity at t + \(\tau\) which is averaged over all data points in given time interval.
As an example, Figure 37 shows how an autocorrelation function is constructed. The example uses simulated data in which the fluctuation is represented by a Gaussian spike. Figure 37a shows the original fluctuation. In autocorrelation analysis, in accordance with equation (4.1), a copy of the fluctuating data is produced and shifted in time by an amount \( \tau \). The product of the original signal and its shifted replicate is the integrand shown in equation (4.2). Each point on the autocorrelation function, Figure 37c, is the result of an integrand of this type. The integral is always greatest when \( \tau = 0 \) and decays in a time scale that is determined by the duration of the fluctuations. In actual FCS data there will be a large number of fluctuations, typically millions, over which this effect is averaged. FCS is in effect a bulk measurement that results from observations of a large number of individual events recorded over time.
Figure 37. Depiction of the formation of an autocorrelation curve.
In an actual FCS experiment, the autocorrelation decay for fluorophores diffusing through an observation volume is defined by equation (4.3), which takes into consideration intersystem crossing.

\[ G(\tau) = \left( 1 + Ae^{-\frac{\tau}{\tau_{\text{triplet}}}} \right) \cdot C \cdot \frac{1}{\left( 1 + \frac{\tau}{\tau_D} \right)} \cdot \frac{1}{\left( 1 + \left( \frac{r_o}{z_o} \right)^2 \left( \frac{\tau}{\tau_D} \right) \right)^{\frac{1}{2}}} \]  

(4.3)

The variable C is inversely proportional to the number of molecules in the observation volume. A is a factor that is proportional to the rate constant of receptor from dark to bright states, and the variables \( r_o \) and \( z_o \) are the radial and axial axes of observation volume. Diffusion time is represented by \( \tau_D \) and the triplet relaxation is represented by the variable \( \tau_{\text{triplet}} \).

The function is used to fit the data in an autocorrelation decay as seen from an actual experiment (Figure 38). From this data, the diffusion coefficients can be obtained. The circles on the curve are from equation (4.2) and the solid line used to fit the data is generated from equation (4.3).
Figure 38. Autocorrelation curve of an actual FCS experiment for a single diffusing molecule.

The rates at which molecules diffuse through the observation volume are dependent on their molecular size. Large molecules diffuse through the observation volume slower than small molecules resulting in slower decays that can be visualized in autocorrelation curves. Therefore, the diffusion coefficient, D, can be obtained from the autocorrelation decays as described by equation (4.4)

\[
D = \frac{kT}{6\pi \eta R} = \frac{r_o^2}{4\tau_D}
\]  

(4.4)
where \( k \) is the Boltzmann’s constant, \( T \) is the temperature, \( \eta \) is the viscosity of the solvent, and \( R \) is the hydrodynamic radius \([57]\).

Intersystem crossing results in a nonfluorescent triplet state \([59]\) that is caused by fluorophores crossing over from the first excited state to the lowest lying triplet state before relaxation into the singlet ground state. The relaxation time for the triplet state is observed in the microsecond time scale of an autocorrelation curve \([59, 60]\) and is caused by fluorophores fluctuating between a light-emitting state and a dark state as schematized by the following chemical relationship where \( k_{01} \) and \( k_{10} \) are rate constants.

\[
\text{Fluorescent, light-emitting state} \xrightarrow{k_{01}} \text{Non-fluorescent, dark state} \xleftarrow{k_{10}}
\]

The relaxation time of the triplet state is determined by the sum of the rate constants as defined by equation (4.5).

\[
\tau_{\text{triplet}} = \frac{1}{(k_{10} + k_{01})} \quad (4.5)
\]

An autocorrelation curve can be divided into sections. The first section is due primarily from the triplet state (1 – 10 \(\mu\)s). The second section is comprised of the longest time component (10 \(\mu\)s – 100 ms) and originates from the diffusion of porphyrin 13 bound to vesicles containing PG. The amplitude of this portion of the curve is determined by the relationship between fraction bound and vesicle concentration, \([V]\). The amplitude is proportional to \(\hat{\rho}/[V]\), in which the vesicle concentration is held constant.
4.2 Fluorescence Correlation Spectroscopy (FCS) of a Charged Receptor with PG Anion

The FCS studies were conducted on porphyrin 13 with synthetic vesicles containing the phospholipids, phosphatidylserine (PS) and PG.

![Diagram of phospholipids and porphyrin](image)

Figure 39. Structures of the phospholipids and porphyrin used in the FCS experiments.

The PG and PS phospholipids are both anionic phospholipids. The use of PS in the experiments was to determine the binding selectivity of the receptor to the vesicle.
Figure 40. Autocorrelation decay curve for receptor 13 with synthetic vesicles containing different percentages of PG.

Figure 40, shows the autocorrelation curve of porphyrin 13 binding to vesicles with different percentages of PG. The amplitude of the slow component is proportional to $f^2/[V]$, where $f$ is the fraction bound. As the concentration of PG increases, the fraction bound increases which results in the increase in amplitude. The slow or longest time component occurs due to slower diffusion of the porphyrin 13 being bound to a much larger vesicle.
Figure 41. Binding constants of PE vesicles doped with various percentages of PS and PG. Calculated from PS or PG concentrations.

Figure 41 shows the changes in binding constants as the percentages of PS and PG change within the vesicles. Binding constants were determined using vesicles that contained 10%, 20%, and 30% PG or PS in PE, and the concentration of porphyrin 13 was kept constant. The binding constant for vesicles containing PG is 18000 ± 4200 M⁻¹ and the binding constant for PS is 11000 ± 2100 M⁻¹.

\[
K = \frac{[\text{Bound Receptor}]}{[\text{Free Receptor}][\text{Free PG}]} = \frac{[\text{Receptor}] \cdot f}{([\text{Receptor}] - [\text{Receptor}] \cdot f) \left( \frac{[\text{PG}]}{2} - [\text{Receptor}] \cdot f \right)}
\]
Binding constants are determined by the equation (4.6). The value [PG]/2 is used because it is assumed that half of the PG is located on the inner leaflet of lipid bilayer and inaccessible to binding [61]. The binding fraction, \( f \), is defined by equation (4.7).

\[
\frac{f}{(1 - f) \left( \frac{[\text{PG}]}{2} - [\text{Receptor}] \cdot f \right)}
\]

\[
f = \frac{[\text{Bound Receptor}]}{[\text{Receptor}]}
\]  \hspace{1cm} (4.7)

The binding constants for the PG and PS synthetic vesicles are summarized in Table 1.

**TABLE 1**

BINDING CONSTANT SUMMARY FOR PG IN THE SYNTHETIC VESICLES

<table>
<thead>
<tr>
<th>Synthetic vesicles consisting of various PG percentages with PE</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent PG</td>
<td>0%</td>
<td>10%</td>
<td>20%</td>
<td>30%</td>
</tr>
<tr>
<td>Binding constants (10^4 M^-1)</td>
<td>0</td>
<td>1.9 ± 0.4</td>
<td>1.9 ± 0.4</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Fraction bound</td>
<td>0</td>
<td>0.06 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>0.13 ± 0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Synthetic vesicles consisting of various PS percentages with PE</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent PS</td>
<td>0%</td>
<td>10%</td>
<td>20%</td>
<td>30%</td>
</tr>
<tr>
<td>Binding constants (10^4 M^-1)</td>
<td>0</td>
<td>1.3 ± 0.3</td>
<td>1.1 ± 0.08</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Fraction bound</td>
<td>0</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>
Determination of binding affinities was calculated using two different approaches. The first approach determined the binding affinity of porphyrin 13 for PG and the second approach determined the binding affinity of porphyrin 13 for the vesicles (scheme 14). Using the first approach, the binding constant (Figure 41) of porphyrin 13 to PG remained constant as the percentages of PG increased which indicates the binding constant does not depend on the concentration of PG.

\[
\text{Receptor} + \text{PG} \rightleftharpoons \text{Receptor} \cdot \text{PG} \\
\text{Receptor} + \text{Vesicle} \rightleftharpoons \text{Receptor} \cdot \text{Vesicle}
\]

Scheme 14. Schemes used for determination of binding constants in FCS.

In this study FCS is used to measure the binding affinity of vesicles that contain PG and the vesicles provide a model for the bacterial membrane. Figure 42 shows the binding affinities of porphyrin 13 for the vesicle does vary according to PG concentration.

In the experiments using vesicles that contained both PS and PG, the total anionic phospholipid composition was maintained at 30% and PE was maintained at 70%. Initially the PS + PG vesicle had a higher binding constant than the PS and PG vesicles because of the higher total anionic phospholipid composition. The binding constant of the PS + PG vesicles is \(9.7 \times 10^7 \pm 1.9 \times 10^7\) M\(^{-1}\). Figure 42, portrays an increase in binding constants as more receptor molecules bind to the vesicles. There is not a significant difference between the binding constants of the PS and PG vesicles which indicates little selectivity.
Figure 42. Binding constants of PE vesicles containing PG, PS, and PG + PS. Calculated from vesicle concentrations.

The summary of the binding constants for vesicles containing the PS + PG phospholipids is given in the Table 2. The binding constants are calculated using vesicle concentration. The binding constants were calculated by equation (4.8).

\[ K = \frac{[\text{Bound Receptor}]}{[\text{Free Receptor}][\text{Vesicles}]} = \frac{f}{(1 - f)[\text{Vesicles}]} \]  

(4.8)
<table>
<thead>
<tr>
<th>Percent PG + PS</th>
<th>0</th>
<th>5% + 25%</th>
<th>10% + 20%</th>
<th>15% + 15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding constants (10^7 M⁻¹)</td>
<td>0</td>
<td>9.3 ± 1.4</td>
<td>9.8 ± 2.1</td>
<td>10.0 ± 2.3</td>
</tr>
<tr>
<td>Fraction bound</td>
<td>0</td>
<td>0.081 ± 0.012</td>
<td>0.086 ± 0.019</td>
<td>0.088 ± 0.020</td>
</tr>
</tbody>
</table>

The table summarizes the binding constants for vesicles containing PS and PG phospholipids, with different percentages of PG and PS, and a constant PE (70%).
CHAPTER 5

CONCLUSION

In order to design more selective and high affinity binding pockets for future porphyrin receptors, the binding motif of the receptor-lipid complex needed to be understood. Without the aid of X-ray crystals, attention was turned to spectroscopy to provide the needed information on the receptor-lipid complex. After characterization of the PG anion, $^1$H NMR titrations and inverse titrations were utilized to provide information on the orientation and how the PG anion was interacting with the receptor protons. Analysis of the NMR experiments indicated that PG was positioned in the binding pocket of the various receptors by the upfield movement of the protons on the lipid head group due to their interaction with the porphyrin ring current. This suggests the hydroxyl groups of the PG anion are in a position that allows for binding with the pickets of the receptors due to their proper alignment. The amide protons of the charged receptor move upfield with the addition of the dihydrogen phosphate anion resulting from the loss of hydrogen bonding with the DMSO solvent. With the displacement of DMSO, the hydroxyl groups of PG form hydrogen bonds with the amide protons causing them to move downfield. The movement of the urea and ammonium protons show that are binding to the phosphate anionic portion of the lipid head group. Binding stoichiometries and binding constants (Table 3) of the neutral and charged receptors were determined by Job plot and $^1$H NMR titration experiments.
TABLE 3
SUMMARY OF $^1$H NMR BINDING STOICHIOMETRIES AND BINDING CONSTANTS

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Binding stoichiometry</th>
<th>$K_a$, M$^{-1}$ (error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1:1</td>
<td>2400 (13%)</td>
</tr>
<tr>
<td>8</td>
<td>1:1</td>
<td>6500 (9%)</td>
</tr>
<tr>
<td>9</td>
<td>Complex</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>Complex</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>Complex</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>1:1</td>
<td>4900 (11%)</td>
</tr>
</tbody>
</table>

ITC experiments provided the thermodynamics of binding. A summary of the binding constants and thermodynamic data is summarized in Table 4.

TABLE 4
SUMMARY OF BINDING CONSTANTS AND THERMODYNAMIC DATA FROM ITC

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_a$, M$^{-1}$ (error)</th>
<th>$\Delta H$, cal/mol (error)</th>
<th>$\Delta S$, cal/mol/$^\circ$C</th>
<th>C–value</th>
<th>[Receptor], mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2100 (5%)</td>
<td>-2830 (3%)</td>
<td>6</td>
<td>6</td>
<td>30.7</td>
</tr>
<tr>
<td>8</td>
<td>3700 (12%)</td>
<td>-1210 (3%)</td>
<td>12</td>
<td>7</td>
<td>16.1</td>
</tr>
<tr>
<td>14</td>
<td>2800 (36%)</td>
<td>691 (13%)</td>
<td>18</td>
<td>11</td>
<td>16.7</td>
</tr>
</tbody>
</table>
The C–value, critical parameter, determines the shape of the binding curve and is calculated from equation (4.9)

\[
c = N \times [\text{Receptor}] \times K_a
\]  

(4.9)

where \( N \) is the binding stoichiometry, \( K_a \) is the binding constant, and \([\text{Receptor}]\) is the millimolar concentration of the receptor. The experimental conditions required high receptor concentrations, but were within the acceptable C–value range. The thermodynamic data of receptors 7 and 8 indicate the binding process is enthalpy and entropy driven. The negative enthalpy values result from the hydrogen bonding of the pickets to the multifunctional head group of PG. The difference in entropy values of receptors 7 and 8 is potentially due to difference in picket sizes above the urea functionality and the release of more solvent molecules when PG binds to receptor 8.

FCS was used to determine the binding affinity of receptor 13 for PG in synthetic vesicles which provided a model for a bacterial membrane. Figure 41 depicts the binding constant as the PG concentration increases. The binding constants (Table 1) remain constant which shows that it is not cooperative binding expressed by receptor 13 and the binding fraction increases relative to the percent increase of lipid which indicates the binding stoichiometry is 1:1 for PG found in vesicles. In experiments performed by Zifan Wang, it was determined from surfactant experiments (CTAT) that the binding did not rely solely on Coulombic interactions. These results support that the binding pocket is complementary to the head group of the PG anion. These results corroborate the 1:1 binding stoichiometries that were determined in solution Job plot analysis.
The binding affinity of receptor 13 for the vesicles and the selectivity for PG was depicted in Figure 42. The binding affinity of receptor 13 with vesicles increases with the increasing PG concentration. The PG + PS curve (blue) maintains an anionic composition of 30% and the curve increases slightly as the PG concentration increases with decreasing PS concentration. The PG + PS curve in the figure illustrates the modest selectivity between the vesicles containing PS and PG due to very slight increase in the binding constant as the PG increases.

If these receptors are to be used as a potential therapeutic, then they need to be able to pass through the cell walls of various bacteria. Preliminary investigations have been conducted using *E. coli* and *Bacillus thuringensis* along with receptor 13. The experimental results have demonstrated that the receptor can pass through the cell wall of the bacteria and bind to PG of the cell membrane. Increasing the incubation times of the receptor with the bacteria increased the amount of receptor able to pass through the cell wall and bind to PG within the membrane of the bacteria.

Future experiments include the synthesis of receptors with various binding pockets that will be complementary to the PG head group by extending the pickets from the ammonium groups in receptor 13. Binding studies in solution will provide insight into the binding stoichiometry and motif of the receptor-lipid complex. The incorporation of charges improves the water solubility of the receptor allowing for future studies with synthetic vesicles and bacterial membranes.
CHAPTER 6

EXPERIMENTAL – MATERIALS AND PROCEDURES

All melting points (Mel-Temp) are uncorrected. All $^1$H NMR spectra were recorded at 400 MHz or 300 MHz and were calibrated to the resonances from CDCl$_3$, DMF-d$_7$, and DMSO-d$_6$. $^{19}$F NMR was referenced to trifluoroacetic acid and $^{31}$P NMR was referenced to phosphoric acid. Structure affirmation was accomplished by HRMS by the Mass Spectrometry Lab at the University of Kansas. All UV spectra were recorded using Hitachi U-2010 and Varian Cary 100 UV-vis spectrophotometers. Column chromatography was carried out on silica gel (Davisil 633). Radial chromatography was performed using a Chromatotron (Harrison Research, Palo Alto, California). ITC was performed using the ITC 200 by MicroCal. The phospholipids 1,2-distearoyl-sn-glycero-3-phospho-sn-1-glycerol sodium salt and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine were purchased from Corden Pharma. 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine sodium salt was purchased from Avanti. TBAPG phospholipid was synthesized in our lab [41]. All solvents used in reactions were dried from CaH$_2$, unless otherwise indicated, and reagents were dried in vacuum desiccator with Drierite. 2-O-Benzylethanolamine and 2-(2-(2-Ethoxyethoxy)ethoxy)ethyl amine were dried using a dean-stark apparatus in benzene. All reactions were carried out in a nitrogen atmosphere. Samples used for analytical experiments were dried in vacuum desiccators for minimum of two days over Drierite.
Job Plot

The preparation for a $^1$H NMR Job plot experiment using compound 8 and TBAPG is given as an example. Dry 8 (8.12 mg) was weighed on a six-point microbalance and added to a 2 mL volumetric flask. Dry TBAPG (5.7 mg) was weighed out in a glove bag (nitrogen atmosphere) into a separate previously tared 2 mL volumetric flask using a four-point balance. The samples were dissolved using DMF-d$_7$ (for neutral receptors). Aliquots of both samples were added to separate NMR tube inside the glove bag as follows in Table 5.

TABLE 5

$^1$H NMR JOB PLOT DATA FOR COMPOUND 8

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of Receptor Solution (μL)</th>
<th>Volume of Anion Solution (μL)</th>
<th>Volume of Pure Solvent (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>200.0</td>
<td>0.000</td>
<td>150</td>
</tr>
<tr>
<td>Tube 2</td>
<td>141.0</td>
<td>59.00</td>
<td>150</td>
</tr>
<tr>
<td>Tube 3</td>
<td>131.1</td>
<td>69.00</td>
<td>150</td>
</tr>
<tr>
<td>Tube 4</td>
<td>121.1</td>
<td>78.90</td>
<td>150</td>
</tr>
<tr>
<td>Tube 5</td>
<td>111.2</td>
<td>88.90</td>
<td>150</td>
</tr>
<tr>
<td>Tube 6</td>
<td>101.2</td>
<td>98.90</td>
<td>150</td>
</tr>
<tr>
<td>Tube 7</td>
<td>91.20</td>
<td>108.9</td>
<td>150</td>
</tr>
<tr>
<td>Tube 8</td>
<td>81.10</td>
<td>118.9</td>
<td>150</td>
</tr>
<tr>
<td>Tube 9</td>
<td>71.10</td>
<td>128.9</td>
<td>150</td>
</tr>
<tr>
<td>Tube 10</td>
<td>61.00</td>
<td>139.0</td>
<td>150</td>
</tr>
</tbody>
</table>
For each NMR tube the sum of the molar equivalents was the same. The molar equivalents of the receptor descended as 1, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, and 0.3. The molar equivalents of the anion increased as 0, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, and 0.7. The $^1$H NMR Job plot experiment was conducted at 30°C. The change in chemical shift of the urea, beta, and aromatic protons (Figure 21) were monitored in relation to tube 1 for neutral receptors. The $^1$H NMR Job plot for compound 14 was preformed following the same experimental procedure with 40% DMSO-d$_6$ in CDCl$_3$. Changes in the chemical shift of the methylene and beta protons were monitored in relation to tube 1 for porphyrin 14 (Figure 31).

$^1$H NMR Titration

Compound 8 and TBAPG are given as an example. Dry compound 8 (2.24 mg) was weighed on a six-point microbalance and added to a 1 mL volumetric flask. Dry TBAPG (7.2 mg) was weighed out in a glove bag (nitrogen atomosphere) into a separate previously tared 1 mL volumetric flask using a four-point balance. DMF-d$_7$ was added to each volumetric flask in order to dissolve the samples. The chemical shift of various protons was monitored as the various increments of the TBAPG solution were added. All additions of TBAPG were added in a glove bag and the NMR titration was conducted at 30°C. The equivalents of receptor and anion are given Table 6. The chemical shifts and concentrations of the receptor and anion were calculated for each anion addition and used to calculate the binding constant. The experimental procedure was the same for compound 14 using 40% DMSO-d$_6$ in CDCl$_3$ as the solvent mixture.
TABLE 6

\( ^1H \text{ NMR TITRATION DATA FOR COMPOUND 8} \)

<table>
<thead>
<tr>
<th>Receptor Equivalence</th>
<th>Anion Equivalence</th>
<th>Volume of Receptor Solution (μL)</th>
<th>Volume of Anion Solution (μL)</th>
<th>Concentration of Receptor (mol/L)</th>
<th>Concentration of Anion (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>500</td>
<td>0.00</td>
<td>1.50 x 10^{-3}</td>
<td>0.00</td>
</tr>
<tr>
<td>1</td>
<td>0.20</td>
<td>0.00</td>
<td>21.3</td>
<td>1.44 x 10^{-3}</td>
<td>2.89 x 10^{-4}</td>
</tr>
<tr>
<td>1</td>
<td>0.40</td>
<td>0.00</td>
<td>21.3</td>
<td>1.39 x 10^{-3}</td>
<td>5.55 x 10^{-4}</td>
</tr>
<tr>
<td>1</td>
<td>0.60</td>
<td>0.00</td>
<td>21.3</td>
<td>1.33 x 10^{-3}</td>
<td>8.01 x 10^{-4}</td>
</tr>
<tr>
<td>1</td>
<td>0.80</td>
<td>0.00</td>
<td>21.3</td>
<td>1.29 x 10^{-3}</td>
<td>1.03 x 10^{-3}</td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.00</td>
<td>21.3</td>
<td>1.24 x 10^{-3}</td>
<td>1.24 x 10^{-3}</td>
</tr>
<tr>
<td>1</td>
<td>1.25</td>
<td>0.00</td>
<td>26.7</td>
<td>1.19 x 10^{-3}</td>
<td>1.49 x 10^{-4}</td>
</tr>
<tr>
<td>1</td>
<td>1.50</td>
<td>0.00</td>
<td>26.7</td>
<td>1.14 x 10^{-3}</td>
<td>1.71 x 10^{-3}</td>
</tr>
<tr>
<td>1</td>
<td>1.75</td>
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ITC

Compound 8 and TBAPG are given as an example. Dry compound 8 (3.15 mg) was weighed on a six-point microbalance and added to a 1 mL volumetric flask. Dry TBAPG (28.4 mg) was weighed out in a glove bag (nitrogen atmosphere) into a separate previously tared 1 mL volumetric flask using a four-point balance. DMF was added to each volumetric flask in order to dissolve the samples.

The receptor and anion solutions were degassed for 15 minutes. The syringe was filled with 40 μL of anion solution. The sample cell was filled with 200 μL of receptor solution, and the reference cell was filled with DMF. The solvent or solvent mixture in the reference cell is the same solvent that is used in the sample cell and syringe with the receptor and anion. The anion solution was added in subsequent aliquots (1-2 μL) until the complete addition of the anion solution. Each consecutive injection would add at every 120 second intervals. Control experiments using PG only were setup under the conditions described above. The sample and reference cells were filled with DMF. The two experiments were subtracted from one another to give the thermodynamic data. Experimental conditions were the same for compound 14 which required the use of a ternary solvent mixture of DMSO:CHCl₃:MeOH (5:4.5:0.5) at 40°C.

5,10,15,20-tetra-(2-nitrophenyl)porphyrin (1) [38]: 2-nitrobenzaldehyde (20.2 g, 133.7 mmol) was dissolved in acetic acid (400 mL, 0.33 M). Distilled pyrrole (9.2 mL, 132.6 mmol) was added drop wise to the solution at reflux. The reaction mixture was allowed to stir for 2 hours at reflux, and then allowed to cool to rt. During cooling, chloroform (50 mL) was added to the solution. When the solution reached rt the solution was filtered through a sinter glass funnel.
The solid was washed with cold acetic acid and chloroform. The crude material was eluted off a grade III alumina gel column (100% CHCl₃) yielding 2.5 g (3.146 mmol, 9.4%) of a purple solid.

5,10,15,20-tetra-(2-aminophenyl)porphyrin (2) [38]: Porphyrin 1 (1.609 g, 2.02 mmol) was dissolved in concentrated HCl (65 mL) under nitrogen. SnCl₂·2H₂O (6.437 g, 28.5 mmol) was added to the solution at room temperature. The reaction mixture was transferred to a preheated oil bath at 70°C and allowed to stir for 1 hour. The solution was allowed to cool to rt, and then neutralized with concentrated NH₄OH (250 mL) to a pH greater than 8. CHCl₃ (200 mL) was added to the solution and it was allowed to stir overnight. The layers were separated and the aqueous layer was extracted with CHCl₃ (4 x 200 mL). The combined organic layer was filtered through celite and concentrated under vacuum to yield a purple solid (1.37g) that was a mixture of atropisomers and regioisomers.

Atropisomerization of 5,10,15,20-tetra-(2-aminophenyl)porphyrin (3) [37, 39]: Benzene (139.68 mL) was added to a three-neck round bottom flask with silica gel (52.856 g). The slurry was kept under a benzene saturated nitrogen gas atmosphere. The slurry was heated to 80°C and allowed to stir for 1 hour. After 1 hour, porphyrin 2 (1.598 g, 2.37 mmol) was added to the slurry and allowed to stir for 20 hrs. The slurry was allowed to cool to rt. The silica gel was washed with 150 mL CHCl₃:ether (1:1) then 300 mL toluene:ether (1:1) on a sinter glass funnel. The sinter glass funnel was transferred to a clean filter flask and the silica gel was then washed with ether/isopropyl amine (3.75% isopropyl amine). The ether/isopropyl amine solution was concentrated under vacuum (no external heat) to yield a purple solid (0.988 g, 1.464 mmol,
2-(2-(2-Ethoxyethoxy)ethoxy)ethyl tosylate (5): Triethyleneglycol monoethylether (10 g, 56.11 mmol), dissolved in THF (20mL), was brought to 0°C in an ice bath, whereupon a solution of sodium hydroxide (4.6 g, 115 mmol) dissolved in water (18.4 mL) was added to the stirred, cold mixture that was kept under nitrogen. A solution of tosyl chloride (13.799 g, 72.38 mmol) dissolved in THF (20 mL) was added drop wise over 15 min, and the reaction mixture was allowed to warm to ambient temperature and stirred for 2 hrs. Diethyl ether (150 mL) was added to the reaction mixture and the two layers were separated. The organic layer was washed with 1M NaOH (3 x 12.5 mL) without shaking, and washed with water (2 x 12.5 mL). The organic layer was dried with Na₂SO₄ and concentrated under vacuum to yield a yellow liquid (15.614 g, 46.972 mmol, 84%). ¹H NMR (400 MHz, CDCl₃) δ 7.78 (d, 2H, J = 8.4 Hz), 7.32 (d, 2H, J = 8.4 Hz), 4.14 (t, 2H, J = 4.8 Hz), 3.66 (t, 2H, J = 4.8 Hz), 3.61-3.53 (m, 8H), 3.49 (quartet, 2H, J = 6.9 Hz), 2.42 (s, 3H), 1.18 (t, 3H, J = 7 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 145.00, 133.173, 130.03, 128.20, 70.96, 70.91, 70.73, 69.99, 69.45, 68.87, 66.85, 21.86, 15.36; HRMS (ESI) calcd for C_{15}H_{24}O₆SNa 355.1191, found (M+Na)^+ 355.1186.

2-(2-(2-Ethoxyethoxy)ethoxy)ethyl amine (6): Compound 5 (3 g, 9.02 mmol) was dissolved in DMF (6.2 mL) in a three-neck round bottom flask under nitrogen, and to this solution was added
potassium phthalimide (2.22 g, 11.99 mmol). The reaction mixture was heated to 110°C and allowed to stir for 3 hrs. After cooling to rt, 38.6 mL of diethyl ether was added to the solution and a resultant precipitate was filtered. The filtrate was washed with 1M NaOH (2 x 13 mL) and water (1 x 13 mL). The organic layer was dried with Na₂SO₄ and concentrated under vacuum. The crude phthalimide was dissolved in 6.4 mL of a hydrazine monohydrate/ethanol (1/1 v/v) mixture and heated to 110°C overnight under nitrogen. The reaction mixture was cooled to RT and extracted with toluene (4 x 20.6 mL). The organic layers were combined and concentrated under vacuum to yield a yellow oil (0.986 g, 5.563 mmol, 62%). ¹H NMR (400 MHz, CDCl₃) δ 3.66-3.47 (m, 12H), 2.84 (t, 2H, J = 5 Hz), 1.59 (s, broad, 2H), 1.19 (t, 3H, J = 7.1 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 73.72, 70.90, 70.80, 70.04, 66.87, 42.03, 15.36; HRMS (ESI) calcd for C₈H₁₀NO₃Na (M+Na)+ 200.1263, found 200.1268.

α,α,α,α-5,10,15,20-tetrakis-(2-N-[(2-phenylmethoxy)ethylureido]phenyl)porphyrin (7):

Dry porphyrin 3 (0.253 g, 0.375 mmol) was dissolved in dry CH₂Cl₂ (178 mL) and dry triethylamine (0.42 mL, 3.01 mmol) in a glove box. Triphosgene (0.167 g, 0.563 mmol) in dry CH₂Cl₂ (6 mL) was added to the reaction mixture and it was allowed to stir at ambient temperature for 1.5 hrs. Dry compound 4 (0.284 g, 1.88 mmol) in dry CH₂Cl₂ (4 mL) was then added via syringe in one portion and the reaction mixture was allowed to stir at ambient temperature for 48 hrs. The solution was washed with water (2 x 75 mL), and the organic layer was dried over Na₂SO₄ and then concentrated under vacuum to yield a purple solid. The crude reaction material was purified by silica gel chromatography eluting with CH₂Cl₂:EtOAc (1:1) to remove the impurities and then ethyl acetate (100%) to elute product. The resulting solid was recrystallized from CH₂Cl₂/hexane to yield a pure purple solid (0.095 g, 0.0687 mmol, 18%).
a,a,a,a,-5,10,15,20-tetrakis-(2-(N-(3,6,9-trioxaundecyl)ureido)phenyl)porphyrin (8): Dry porphyrin 3 (0.192 g, 0.285 mmol) was dissolved in dry CH₂Cl₂ (137.2 mL) in a glove box. To the solution was added dry triethylamine (0.32 mL, 2.27 mmol) followed by the addition of triposhogene (0.127 g, 0.427 mmol). The mixture was allowed to stir for 1 hr. at rt. Dry compound 6 (0.262 g, 1.478 mmol), dissolved in dry CH₂Cl₂ (5 mL), was added to the reaction mixture via syringe in one portion, and the reaction was allowed to stir for 24 hrs. The reaction mixture was then concentrated under vacuum, and the resulting crude material was purified using silica gel chromatography eluting with CH₂Cl₂:ethanol (96:4). The product was recrystallized with CH₂Cl₂:hexanes to yield a pure purple solid (0.125 g, 0.084 mmol, 30%). Mp:173-175°C; UV/vis CHCl₃ λ max (lnε): 422 (12.14), 516.5 (9.62), 545 (8.72), 590 (8.54), 648 (7.78); ¹H NMR (400 MHz, DMSO) δ 8.73 (s, 8H), 8.45 (d, 4H, J = 8.4 Hz), 7.72 (t, 4H, J = 7.9 Hz), 7.60 (d, 4H, J = 7.6 Hz), 7.36 (s, broad, 4H), 6.21 (t, broad, 4H), 3.40-3.13 (m, 48H), 2.94-2.86 (m, 8H), 0.98 (t, 12H, J = 7 Hz), -2.68 (s, broad, 2H); ¹³C NMR (100 MHz, DMSO) δ 155, 140, 135, 130, 129, 120.9, 120.8, 116, 69.6, 69.5, 69.4, 69.2, 69, 65, 15; HRMS calcd for C₈₄H₇₉N₁₂O₈(M+H)⁺ 1383.6144, found 1383.6151; HRMS (ESI) calcd for C₈₄H₇₈N₁₂O₈Na(M+Na)⁺ 1405.5963, found 1405.5918.
α,α,α,α-5,10,15,20-tetrakis-(2-((N-(2-hydroxyethyl)ureido)phenyl)porphyrin (9) : To a reaction mixture containing porphyrin 7 (0.165 g, 0.119 mmol) dissolved in ethanethiol (22.14 mL, 309 mmol) was added BF₃·etherate (0.83 mL, 6.749 mmol). The reaction was kept under nitrogen and allowed to stir at rt for 4 hrs. At this time the reaction was quenched with a saturated solution of NaHCO₃, and the mixture was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and precipitate was collected from the extractions was filtered. The resultant crude solid was dissolved in DMF and added to a Chromatogram plate and allowed to air dry overnight. The product was purified using radial chromatography eluting with CH₂Cl₂:methanol:water (84:15:1) resulting in a pure purple solid (0.031 g, 0.030 mmol, 25%). Mp: 218°C, dec; UV/vis DMF λ max (lnε): 422.5 (14.31), 515 (11.91), 550 (10.66), 590 (10.76), 647 (10.13); ¹H NMR (400 MHz, DMSO) δ 8.73 (s, 8H), 8.48 (d, 4H, J = 8 Hz), 7.72 (t, 4H, J = 7.9 Hz), 7.61 (d, 4H, J = 7.6 Hz), 7.38 (s, broad, 4H), 7.29 (t, 4H, J = 7.4 Hz), 6.16 (t, broad, 4H), 4.35 (s, broad, 4H), 3.10-3.09 (m, 8H), 2.81-2.80 (m, 8H), -2.66 (s, broad, 2H); ¹³C NMR (100 MHz, DMSO) δ 155, 140, 135, 130, 129, 120.7, 120.6, 116, 60, 42; HRMS (ESI) cald for C₅₆H₅₅N₁₂O₈ (M+H)⁺ 1023.4266, found 1023.4265; HRMS (ESI) cald for C₅₆H₅₄N₁₂O₈Na (M+Na)⁺ 1045.4085, found 1045.4169.

α,α,α,α-5,10,15,20-tetrakis-(2-(N-(propylureido))phenyl)porphyrin (10): To a solution of dry porphyrin 3 (0.413 g, 0.612 mmol) dissolved in dry CH₂Cl₂ (12.2 mL) was added propyl
isocyanate (0.459 mL, 4.897 mmol) via syringe in one portion, and the reaction mixture was allowed to stir overnight in a glove box. The solution was removed from the box, and then washed with water (3 x 25 mL) and with brine (1 x 50 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated under vacuum. The crude product was purified using silica gel chromatography eluting with CH$_2$Cl$_2$:EtOAc (20:80). The resultant solid was recrystallized from CH$_2$Cl$_2$:hexanes to yield a pure purple solid (0.126 g, 0.124 mmol, 20%). Mp: 232°C, dec; UV/vis CHCl$_3$ λ max (lnε): 422 (12.12), 516 (9.51), 554 (8.40), 590 (8.45), 646 (7.86); $^1$H NMR (400 MHz, DMSO) δ 8.74 (s, 8H), 8.35 (d, 4H, J = 8.8 Hz), 7.77-7.72 (m, 8H), 7.35 (t, 4H, J = 11.3 Hz), 6.95 (s, broad, 4H), 5.89 (t, broad, 4H), 2.64 (quartet, 8H, J = 6.4 Hz), 1.01 (sextet, 8H, J = 7.3 Hz), 0.50 (t, 12H, J = 7.4 Hz), -2.685 (s, broad, 2H); $^{13}$C NMR (100 MHz, DMSO) δ 155, 140, 135, 131, 129, 121.5, 121.1, 116, 41, 23, 11; HRMS (ESI) cald for C$_{60}$H$_{63}$N$_{12}$O$_4$ (M+H)$^+$ 1015.5095, found 1015.5139; HRMS (ESI) cald for C$_{60}$H$_{62}$N$_{12}$O$_4$Na (M+Na)$^+$ 1037.4915, found 1037.4960.

$[a,a,a,a,-5,10,15,20$-tetrakis-(2-N-(propylureido))phenyl]porphinato]zinc (II) (11):
Porphyrin 10 (0.043 g, 0.041 mmol) was degassed for 2 hours in CH$_2$Cl$_2$ (21.4 mL). To the solution was added Zn(acac)$_2$ (0.033 g, 0.126 mmol). The reaction mixture was allowed to stir at rt for 5 hours. The solvent was removed under vacuum. The resulting material was extracted with ether until no color could be observed. The ether was removed under vacuum and then recrystallized in CH$_2$Cl$_2$/hexane. The resulting product was a purple solid (0.034 g, 0.031 mmol, 74.94%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ 8.678 (s, 8H), 8.344 (d, 4H, J = 8.4 Hz), 7.850 (d, 8H, J = 9.2 Hz), 7.717 (t, 4H, J = 8.6 Hz), 6.547 (s, broad, 4H), 5.715 (s, broad, 4H), 2.560 (quartet, broad, 8H), 0.903 (sextet, 8H, J = 7.3 Hz), 0.439 (t, 12H, J = 7.4 Hz); $^{13}$C NMR (100
MHz, DMSO-d₆) δ 155.046, 139.728, 135.078, 130.867, 129.092, 121.521, 121.058, 115.679, 40.671, 22.509, 11.030.

α,α,α,α-5,10,15,20-tetrakis-(2-(N-[(2-t-butylcarbamoyl)ethanamide]phenyl)porphyrin (12): Under a nitrogen gas atmosphere, dry porphyrin 3 (0.3235 g, 0.479 mmol, 1 equiv.) and BOC-Gly-OH (0.3779 g, 2.157 mmol, 4.5 equiv.) were dissolved in dry CH₂Cl₂ (8.7 mL) and the solution was transferred to an ice bath. EDAC•HCl (0.3676 g, 1.918 mmol, 4 equiv.) in dry CH₂Cl₂ (6.5 mL) was added to the reaction mixture and it was allowed to stir overnight in a dewar at 0°C. The solution was washed with water (2 x 25 mL) and the organic layer was dried with Na₂SO₄. The solution was filtered and concentrated under reduced pressure. The product was purified using column chromatography eluting with EtOAc:CH₂Cl₂ (1:1) to yield a purple solid (0.3196 g, 0.2452 mmol, 62%). Mp: 186 – 188°C; UV/vis DMF λ max (lnε): 423.0 (12.72), 516.0 (10.07), 549.0 (9.18), 590.0 (9.13), 655.0 (8.72); ¹H NMR (400 MHz, DMSO) δ 8.82 (s, broad, 4H), 8.69 (s, 8H), 8.14 (d, 4H, J = 8.4 Hz), 7.93 (d, 4H, J = 6.8 Hz), 7.84 (t, 4H, J = 7.8 Hz), 7.58 (t, 4H, J = 7.8 Hz), 6.44 (s, broad, 4H), 2.83 (s, 8H), 1.001 (s, 36H), -2.77 (s, 2H); ¹³C NMR (100 MHz, DMSO) δ 168.2, 155.4, 137.9, 136.0, 134.5, 131.1, 129.2, 124.7, 124.0, 115.6, 77.9, 42.9, 27.8; HRMS (ESI) calcd for C₇₂H₇₈N₁₂O₁₂(M+H)⁺ 1303.5940, found 1303.6008; HRMS (ESI) calcd for C₇₂H₇₈N₁₂O₁₂Na(M+Na)⁺ 1325.5760, found 1325.5718.

α,α,α,α-5,10,15,20-tetrakis-(2-(N-[(2-ammonium)ethanamide]phenyl)porphyrin tetrakistrifluoroacetate (13): Porphyrin 12 (0.6233 g, 0.478 mmol, 1 equiv.) was dissolved in CH₂Cl₂ (11.8 mL) and transferred to an ice bath. TFA (11.8 mL) was added to the solution and
the reaction mixture was allowed to stir overnight in an ice bath in a dewar. The reaction mixture was concentrated under reduced pressure. The crude solid was triturated with diethyl ether to remove excess TFA. Residual solvent was removed under reduced pressure to yield a purple solid which was dried with a drying pistol at 120°C (0.797 g, 0.586 mmol, 62%). Mp: 201 – 203°C; UV/vis DMF λ max (lnε): 428.5 (11.29), 521.0 (9.32), 553.5 (8.12), 593.5 (8.19), 659.0 (7.81); 1H NMR (400 MHz, DMSO) δ 9.63 (s, broad, 4H), 8.64 (s, 8H), 8.27 (d, 4H, J = 8.4 Hz), 7.80-8.00 (m, 20 H), 7.58 (t, 4 H, J = 7.4 Hz), 2.85 (s, 8H), -2.74 (s, 2H); 13C NMR (100 MHz, DMSO) δ 165.3, 158.3, 137.4, 135.8, 134.2, 130.8, 129.3, 124.3, 124.2, 117.7, 115.3, 114.8, 76.7; 19F (376 MHz, DMSO) δ -75.521 (s, 12F); HRMS (ESI) calcd for C52H48N12O4 (M+2H)⁺/2 452.1961, found 452.1940.

α,α,α,α-5,10,15,20-tetrakis-(2-(N-[[(2-ammonium)ethanamide]phenyl)porphyrin tetrakishexafluorophosphate (14): Ammonium hexafluorophosphate (1.108 g, 6.80 mmol, 20 equiv.) was dissolved in EtOAc (37.8 mL) and added to porphyrin 13 (0.4393 g, 0.340 mmol, 1 equiv.). The reaction mixture was allowed to stir overnight at rt. Water (17 mL) was added to the reaction mixture and allowed to stir for 30 min. The layers were separated and the organic layer was washed water (2 x 17 mL), dried with Na2SO4, filtered and concentrated under reduced pressure. To the product (0.4067 g) was added ammonium hexafluorophosphate (1.026 g, 6.29 mmol, 20 equiv.) dissolved in EtOAc (35 mL). The reaction mixture was allowed to stir at rt overnight. Water (16 mL) was added to the reaction mixture and it was allowed to stir for 30 min. The layers were separated and the organic layer was washed with water (2 x 16 mL), dried with Na2SO4, filtered and concentrated under reduced pressure to yield a purple solid (0.2184 g, 0.1469 mmol, 51%). Mp: 270°C, dec; UV/vis DMF λ max (lnε): 421.0 (16.47), 517.0 (13.63), 659.0 (7.81); 1H NMR (400 MHz, DMSO) δ 9.63 (s, broad, 4H), 8.64 (s, 8H), 8.27 (d, 4H, J = 8.4 Hz), 7.80-8.00 (m, 20 H), 7.58 (t, 4 H, J = 7.4 Hz), 2.85 (s, 8H), -2.74 (s, 2H); 13C NMR (100 MHz, DMSO) δ 165.3, 158.3, 137.4, 135.8, 134.2, 130.8, 129.3, 124.3, 124.2, 117.7, 115.3, 114.8, 76.7; 19F (376 MHz, DMSO) δ -75.521 (s, 12F); HRMS (ESI) calcd for C52H48N12O4 (M+2H)⁺/2 452.1961, found 452.1940.
$^1$H NMR (400 MHz, DMSO) δ 9.66 (s, broad, 4H), 8.73 (s, 8H), 8.26 (d, 4H, J = 8 Hz), 7.92 (t, 8H, J = 7.8 Hz), 7.78 (s, broad, 12H), 7.55-7.65 (m, 4H), 2.96 (s, 8H), -2.74 (s, 2H); $^{13}$C NMR (100 MHz, DMSO) δ 165.5, 136.76, 136.67, 134.4, 131.3, 129.4, 124.84, 124.63, 115.7, 40.5; $^{19}$F (376 MHz, DMSO) δ -71.549 (d, 24F, J = 711.4 Hz); $^{31}$P (161 MHz, DMSO) δ 107.945 (septet, 4P, J = 707.4 Hz); HRMS (ESI) calcd for C$_{52}$H$_{47}$N$_{12}$O$_4$ (M+H)$^+$ 903.3843, found 903.3878; calcd for C$_{52}$H$_{48}$F$_6$N$_{12}$O$_4$P (M+PF$_6$+2H)$^+$ 1049.3485, found 1049.3684; calcd for C$_{52}$H$_{49}$F$_{12}$N$_{12}$O$_4$P$_2$ (M+2PF$_6$+3H)$^+$ 1195.3283, found 1195.3276; calcd for C$_{52}$H$_{50}$F$_{18}$N$_{12}$O$_4$P$_3$ (M+3PF$_6$+4H)$^+$ 1341.3003, found 1341.3066.

**Synthetic membranes [62]**: PE, PS, and PG were mixed in various compositions that had a total mass of 35 mg. The lipid mixture was dissolved in 15 mL CHCl$_3$:MeOH:H$_2$O (65:35:8). The solvent was removed under reduced pressure. A HEPES (25mM):Na$_2$SO$_4$ (50mM) buffer solution was titrated with aqueous NaOH to a pH of 6.5 was prepared and 10 mL of this buffer solution was added to the resulting residue under a nitrogen gas atmosphere. The solution was heated to 85°C until the residue completely hydrated. Unilamellar vesicles were extruded four times, while hot, with a mini-extruder and 200 nm polycarbonate membranes.
REFERENCES
REFERENCES


APPENDIX
Figure 43. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 3.
Figure 44. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 5.
Figure 45. HRMS data for compound 5.
Figure 46. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 6.
Figure 47. HRMS data for compound 6.
Figure 48. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 7.
Figure 49. HRMS data for compound 7.
Figure 50. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 8.
Figure 51. HRMS data for compound 8.
Figure 52. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 9.
Figure 53. HRMS data for compound 9.
Figure 54. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 10.
Figure 55. HRMS data for compound 10.
Figure 56. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 11.
Figure 57. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 12.
Figure 58. HRMS data for compound 12.
Figure 59. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 13.
Figure 60. $^{19}\text{F}$ NMR data of compound 13.
Figure 61. HRMS data for compound 13.
Figure 62. $^1$H NMR (top) and $^{13}$C NMR (bottom) data for compound 14.
Figure 63. $^{19}$F NMR (top) and $^{31}$P NMR (bottom) data for compound 14.
Figure 64. HMRS data for compound 14.
Figure 65. 300 MHz $^1$H NMR data of TBAPG in DMF-d$_7$. 
Figure 66. COSY NMR data for TBAPG.
Figure 67. HMQC NMR data for TBAPG.