Spring 4-2010

The Development of an Effective Water-Soluble Receptor for Pyrene Derivative Dyes

Ashley Longstreet
Lynchburg College

The Development of an Effective Water-Soluble Receptor for Pyrene Derivative Dyes

Ashley Longstreet

Senior Honors Project

Submitted in partial fulfillment of the graduation requirements of the Westover Honors Program

Westover Honors Program

April 2010

Dr. Priscilla Gannicott **Committee chair**

Dr. Nancy Cowden

Dr. David Freier

Dr. Dwight Williams

Abstract

A receptor with enhanced water solubility was synthesized from an existing receptor to be used as a pyranine fluorescence quencher in endovesiculation detection assays. The existing receptor consisted of a cyclen core with four protruding arms containing an aryl nitro group at each end. Its poor water solubility limited the accuracy and precision of the endovesiculation assay because the low concentration of receptor did not match the concentration of pyranine. To increase the water solubility, ethoxy groups were attached to the ends of each arm by first selectively reducing the nitro group to an amine with $NaBH_4$, H_2, and a Raney nickel slurry, producing a 19% yield. A nucleophilic substitution was then performed to attach a 2-ethoxyethyl group to each amine group. Even though the new receptor's maximum concentration in water increased, the ability to quench the fluorescence of pyranine decreased dramatically, thus preventing it from being useful in the endovesiculation detection assays.

Introduction

Biomembrane Assays

Biomembrane assays serve a role in monitoring different cellular membrane functions, such as membrane leakage, lipid flip, and endovesiculation.[1,2] When developing new methodologies for drug delivery and function, biomembrane assays can be an important tool for monitoring the effectiveness of the new delivery systems and the effectiveness of the drugs.[1,2] For these assays to be meaningful it is important to develop accurate and precise methods. This can often be done using liposomes as model systems of the living cells.[1,2] Liposomes are model spherical bilayers which mimic a cell's phospholipid bilayer found in the membrane.[1] Refer to Figs. 1, 2, and 3 for liposomes in the different biomembrane assays. Along with the liposomes, the biomembrane assays require a fluorescent molecule or dye, a molecule that can quench the dye to prevent it from fluorescing under near-physiological conditions, and a fluorometer to measure the difference in fluorescence resulting after the quench.

One of the assays commonly used to monitor the dynamic actions of the cell membrane is the membrane leakage assay. This assay is used to assess ionophores that may be a new basis for an array of different medications such as antibiotics and therapeutics for genetic diseases.[1] Ionophores may also serve as a basis to sensors and enzyme mimics.[1] Ionophores are substances that allow ions to transport across the lipid membrane such as the peptides melittin or gramicidin.[1] In the development of the membrane leakage assay, liposomes are prepared containing a fluorescent dye such as the common pyrene derivative dye pyranine.[1] The ionophore and a fluorescent quencher are then added simultaneously.[1] The ionophores allow the pyranine to leak out of the liposomes, and the quencher will prevent any dye found outside of the

liposomes from fluorescing.[1] The difference in fluorescence intensity of any pyranine trapped in the liposomes can then be measured by a fluorometer.[1]

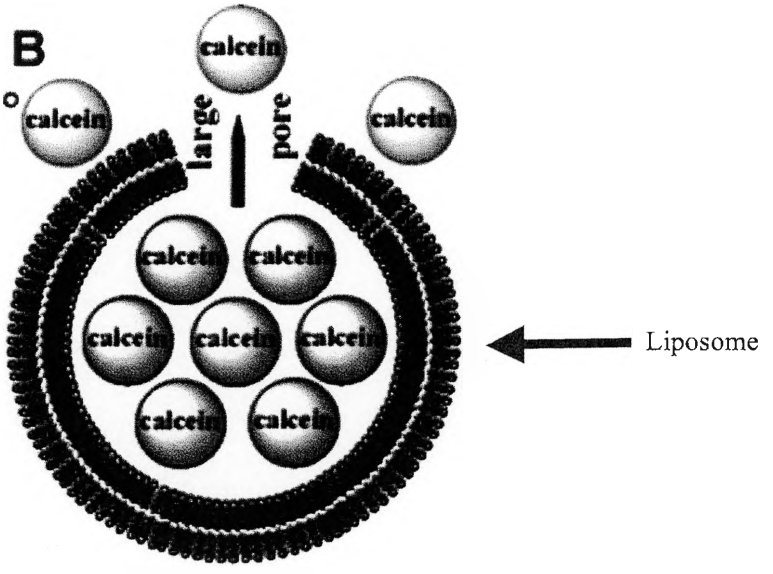


Figure 1. Diagram representing the membrane leakage assay.[1] The pyrene derivative dye calcein is shown leaking out of the liposome.[1] The large pore is created by an ionophore, and it allows calcein to leak outside of the liposome.[1] A quencher would quench the fluorescence of any calcein found outside of the liposome, and the fluorescence intensity of any dye remaining inside the liposome would be measured by a fluorometer.[1]

Another method that is used to monitor cell membrane activity is the lipid flip detection assay. This assay can be used to test the effectiveness of certain molecules proposed to mediate lipid flip from one side of the membrane to the other.[2] Also, this assay observes flippase, floppase, and scramblase activity in live cells.[2,3] The enzymes flippase, floppase, and scramblase act as lipid transporters.[3] Flippases transport phospholipids of a cellular membrane from facing outside the cell to inside the cell, floppases transports phospholipids from facing inside the cell to outside the cell, and scramblases can transport phospholipids in any direction.[3] The lipid flip detection assay is performed by preparing liposomes containing a significant amount of fluorescently tagged phospholipids that contain a fluorescent molecule at the head of a phospholipid.[2] A synthetic molecule proposed to mediate lipid flip is added.[2] After a certain

amount of time, a fluorescent quencher is placed outside of the liposomes, preventing any fluorescently tagged phospholipids facing outside of the liposome from fluorescing.[2] The fluorometer can then measure the fluorescence intensity of the tagged phospholipids facing inside of the liposome.[2] Changes in the fluorescence can indicate whether or not lipid flip is occuring.

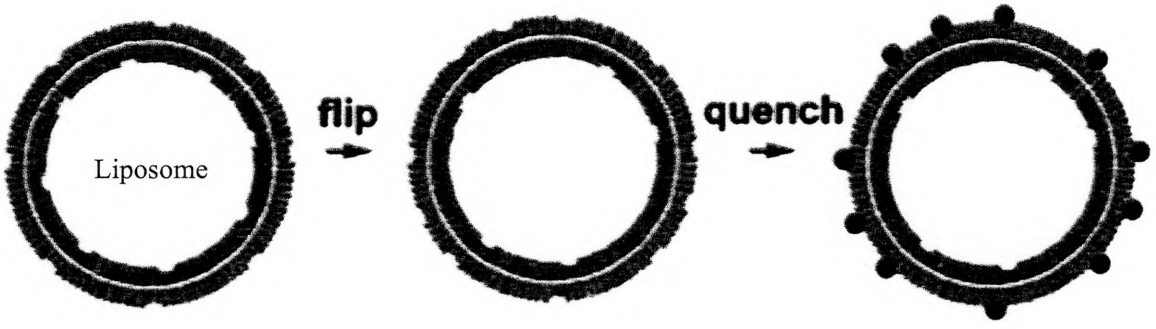


Figure 2. Diagram representing the lipid flip detection assay.[2] The yellow dots represent the fluorescent tag on the fluorescently tagged phospholipids in a liposome.[2] The fluorescently tagged lipids would migrate in and out of the cell by any synthetic molecule capable of doing so.[2] A fluorescence quencher is then added to the solution to keep the fluorescent tags from fluorescing.[2] The fluorescence intensity of any fluorescent tags facing inside the cell or liposome can then be measured with the fluorometer.[2]

The third assay that is commonly used to monitor cell membrane activity is the endovesiculation assay. Endovesiculation is the process in which a vesicle intakes different materials that are too large to freely move past the membrane or too polar to pass through the hydrophobic center of the membrane.[4] Developing a proper method to observe endovesiculation can lead to the development of a method to observe endocytosis in cells. Endocytosis only differs from endovesiculation by being the process in which a live cell intakes material vital to cellular function, and it is induced by different membrane proteins.[4] To induce endovesiculation without the assistance of membrane proteins small molecules used in anesthetics such as chlorpromazine (CPZ) are used.[4] In an endovesiculation detection assay as shown in Fig. 3, the fluorescent dye pyranine is added to the solution outside of the liposomes.[1] Chlorpromazine is added to induce endovesiculation and allow the liposomes to uptake the pyranine.[1] After a certain period of time, any pyranine remaining outside of the liposome will be quenched by

adding the quencher to the solution. The fluorescence of the dye inside the liposome can then be measured.[1]

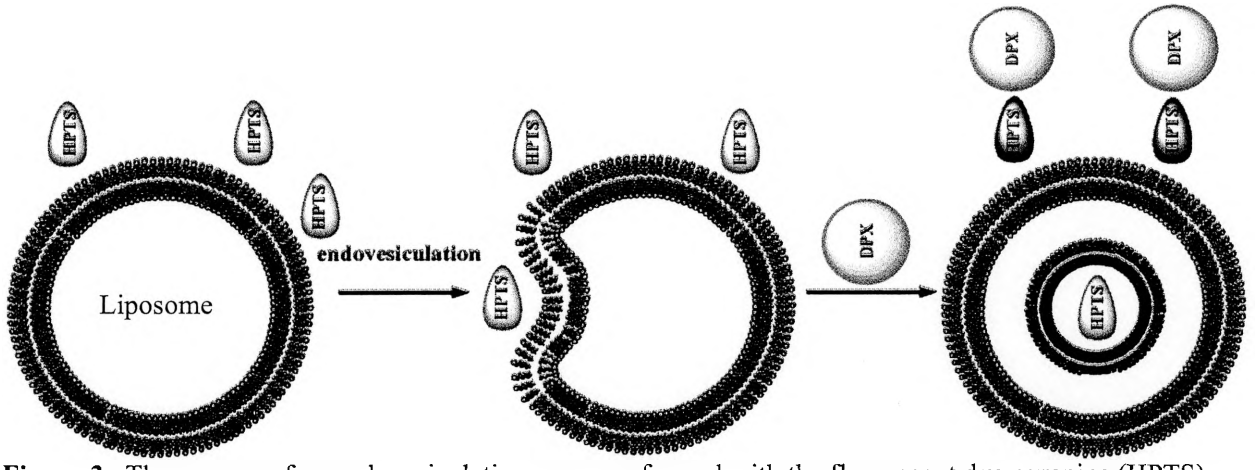


Figure 3. The process of an endovesiculation assay performed with the fluorescent dye pyranine (HPTS) and the quencher DPX.[1] Pyranine is placed outside of the liposomes, and endovesiculation is induced by CPZ.[1] This diagram shows endovesiculation occuring by the liposome's membrane folding around pyranine as pyranine enters the cell.[1] The quencher DPX is added to the outside of the liposome to stop the pyranine outside of the liposome from fluorescing and allow the pyranine inside the liposome to fluoresce.[1] The fluorescence intensity of the pyranine inside the liposome is then measured.[1]

Quenchers and Problems Associated with Each

The common feature among each of the assays mentioned above is the need for a compound or molecule that can quench the fluorescence of the pyranine or the fluorescently tagged phospholipid.[1,2] For a quencher to be viable for use in biomembrane assays, it must be impermeable to the lipid membrane, effectively quench fluorescence at low concentrations, and not alter the membrane in any way.[1,2] Without a viable quencher, the biomembrane assays will not work effectively. One quencher shown to fulfill these requirements for the membrane leakage and lipid flip detection assays is the receptor cyclen 1.[1,2]

Receptors such as cyclen 1 are quenchers that cause a fluorescence quench by forming a complex with the pyrene-derivative dyes as shown in Fig. 4.[1] Some of the unique features include a cyclen core with four nitrogens. These nitrogen atoms allow for arms which contain an ester and a thiourea or urea group to be attached.[1] In the absence of sodium ions, the arms are

relatively free moving. However, in the presence of sodium ions, the ester groups form a chelate which lock the arms in place. Once locked in place the thiourea or urea group can form a hydrogen bond with the fluorescent dye resulting in the fluorescence quench.[1] In addition to the hydrogen bonding, cyclen 1 contains a naphthalene group that can pi stack with the dye.[1]

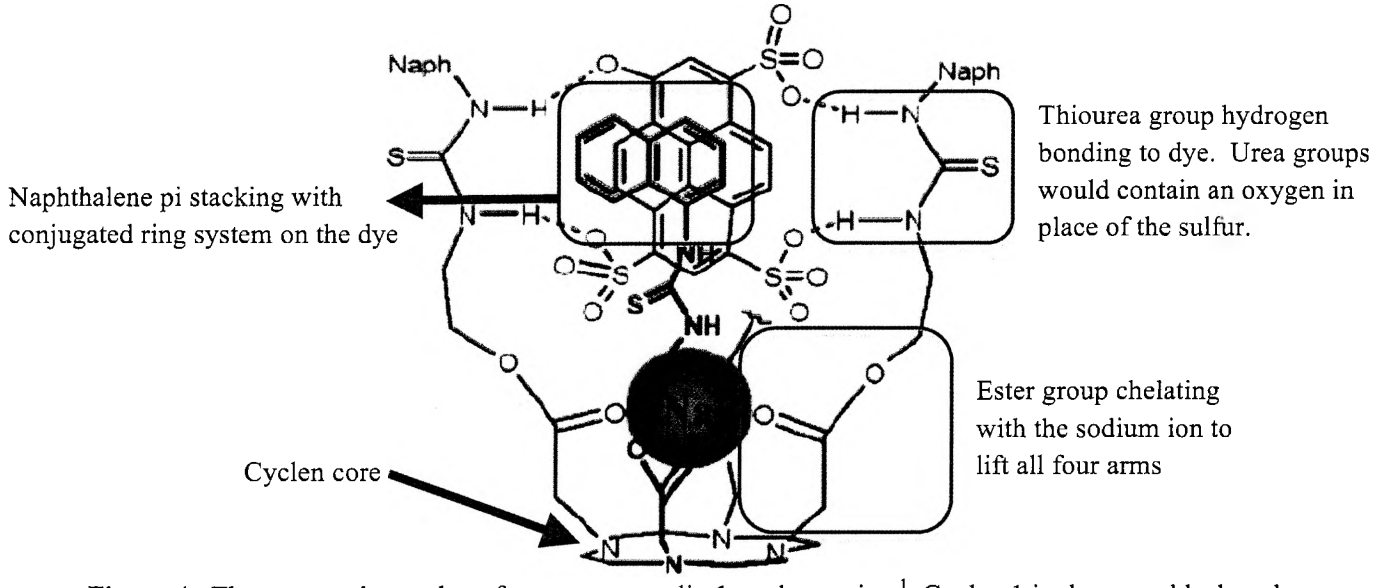


Figure 4. The proposed complex of a receptor, cyclin 1, and pyranine.[1] Cyclen 1 is shown as black and the dye is red. The thiourea or urea and the ester are key functional groups in the formation of the complex.[1] The thiourea or urea is involved with forming the complex with the pyrene derivative dye, and the ester brings up each arm to wrap around the dye.[1] There may be pi stacking involved between the naphthalene and the conjugated ring system of the dye.[1]

Cyclen 1 and another quencher *p*-xylene-bis-pyridinium bromide (DPX) have been used in endovesiculation assays.[1,4] However, neither cyclen 1 or DPX work well in this assay. The problem with cyclen 1 is that it has very limited solubility in water, and biomembrane assay must be performed in solutions mimicking physiological conditions.[1] In endovesiculation assays high concentrations of dye are found outside of the liposomes that need to be quenched, and the highest possible concentration of cyclen 1 in water cannot mask the concentration of the dye.[1] Also, a cyclen 1 solution in dimethyl sulfoxide (DMSO) must be used to insert the cyclen 1 into all three assays.[1,2] Dissolving cyclen 1 in DMSO first, then inserting it in very small quantities

into the assays will allow the maximum concentration of cyclen 1 needed in the assay.[1,2] Very small, microliter quantities of this cyclen 1 in DMSO solution can be used effectively in the membrane leakage and lipid flip detection assay without altering the membranes.[1,2] However, the amount of the DMSO solution needed for the endovesiculation assay is enough to alter the membrane permeability and prevent accurate and precise results of the assay.[1] The quencher DPX is also not viable in endovesiculation assays because it has been shown to alter the fluidity, the bilayer transition phase temperatures, and size of the vesicles at the high concentrations needed in the assay.[1,4]

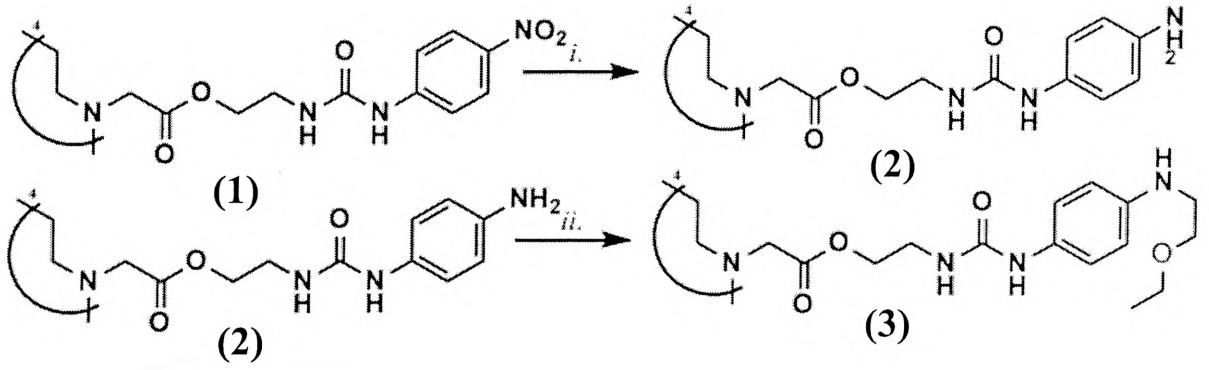
Chemistry

Synthesizing a receptor with increased water solubility

As stated earlier a major problem with cyclen 1 is its limited solubility in water. Other receptors produced by the Sidorov group at Virginia Commonwealth University have the same problem. A popular method used to increase the water solubility of different compounds is to attach an ethoxy chain to the compound.[5] One of the receptors that quenches pyrene-derivative dyes well but has a very low solubility in water is the nitro receptor (**1**) shown in scheme 1. It was thought that by attaching the ethoxy chain to this derivative through the reaction sequence shown in scheme 1 to give the ethoxy receptor (**3**) would improve the overall water solubility. The addition of the ethoxy group should not cause any interference of the receptor binding to the pyrene derivative dyes because it is added to the end of each arm, away from where the complex is formed between the receptor and the dye.

The ideal synthetic route to the new ethoxy derivative is shown in Scheme 1 and begins with the reduction of the nitro group of the nitro receptor (**1**) to the amine in the amine receptor (**2**). The amine in the amine receptor (**2**) can then be reacted with 2-ethoxyethyl 4-

methylbenzenesulfonate (**7**) (refer to scheme 3) to give the final ethoxy receptor (**3**). The entire ethoxy receptor (**3**) is shown in Fig. 5 with all four arms.



Scheme 1. The receptor reaction scheme with the original nitro receptor (**1**), the amine receptor (**2**), and the final ethoxy receptor (**3**). Only one of the four arms is shown in each compound. *i.* $NaBH_4$, Raney nickel slurry, and supplemental H_2 gas in MeOH at 35-45° C for 4 hours. *ii.* 2-ethoxyethyl *p*-toluenesulfonate and K_2CO_3 in DMF at 50° C for 72 hours.

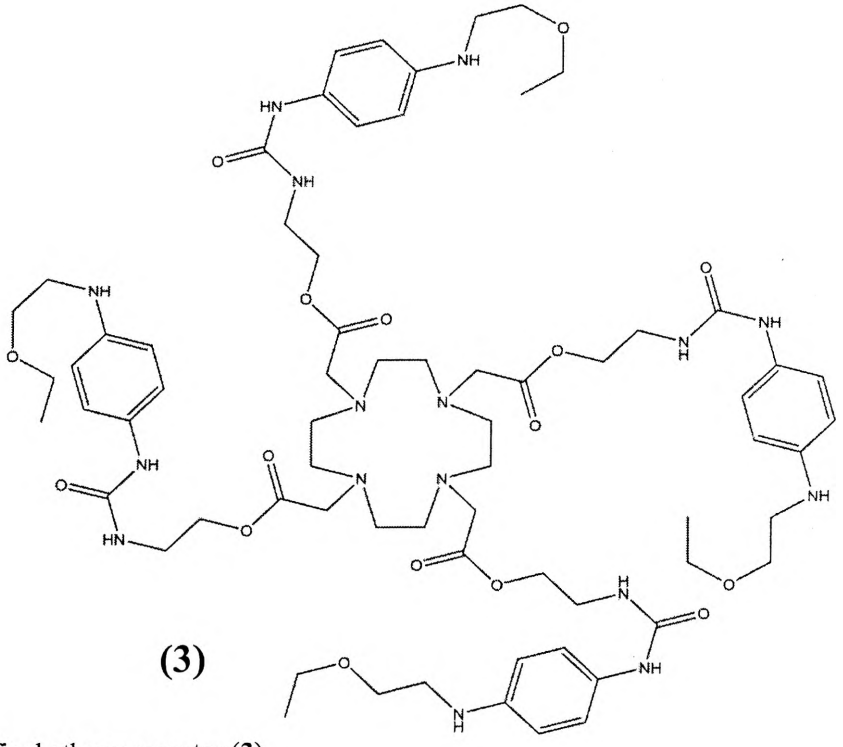
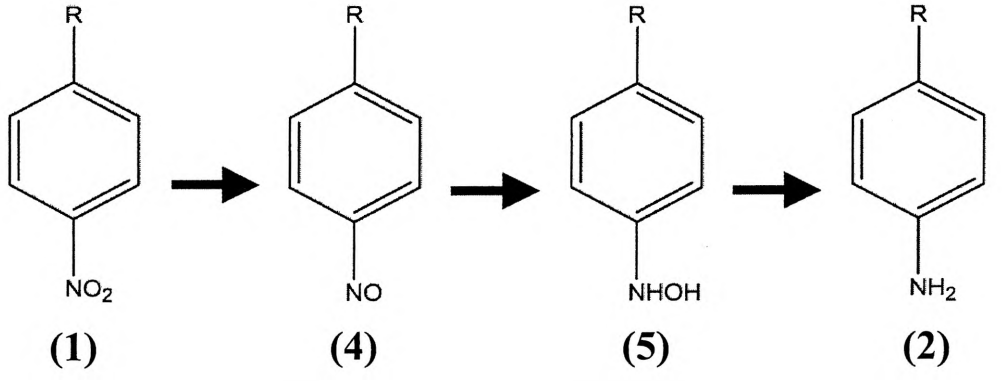


Figure 5. The final ethoxy receptor (**3**).

To attach the ethoxy group onto each arm, the nitro group of the nitro receptor (**1**) must first be reduced to the amine in the amine receptor (**2**). Reduction of the nitro groups must be

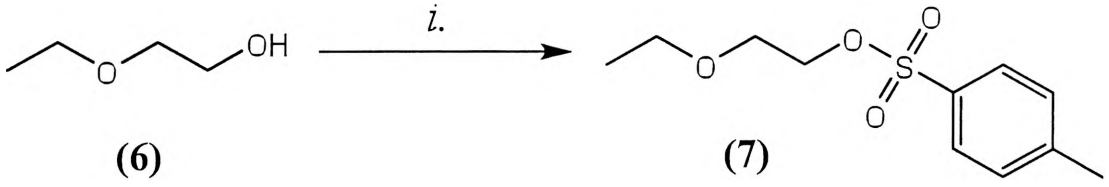
selective due to the ester and urea groups present. The urea and ester groups serve a valuable purpose by forming the complex with the dye. The quenching ability of the receptor would be altered if these functional groups are altered in any way. If the ester functional groups are reduced, the arms of the receptor would detach from the cyclen core. Thus, a selective method to carry out this reduction is key. Among the more common methods for the reduction of arylnitro groups are palladium on a carbon catalyst (Pd/C) with hydrogen gas, iron and hydrochloric acid, and tin and hydrochloric acid.[6] Because the acidic conditions of the latter two methods are expected to damage the ester and urea functional groups, coupled with the high cost of the Pd/C, another method was chosen for this research. A method using sodium borohydride ($NaBH_4$) in the presence of a Raney nickel catalyst has previously been shown to reduce an arylnitro in the presence of esters, amides, and other functional groups.[7]

One of the major concerns when reducing arylnitro groups is the formation of unwanted byproducts. These byproducts are a result of the intermediates that can be formed when reducing the arylnitro groups. The intermediates produced in the course of reducing a nitro group are the nitroso (**4**) and hydroxylamine (**5**) (refer to Scheme 2). This reaction is further complicated in this research as four nitro groups must be reduced simultaneously and completely.[7]



Scheme 2. The end of an arm of the nitro receptor (**1**) and the intermediates that occur before reaching the full reduction into the amine (**2**). The nitro is reduced into the nitroso (**4**), hydroxyamine (**5**), and the amine (**2**) respectively.

Once the reduction of the nitro group is achieved, the next task is the attachment of the ethoxy group onto the amine receptor. This reaction is anticipated to be accomplished via an S_N2 reaction between the arylamine in the amine receptor (**2**), and 2-ethoxyethyl 4-methylbenzenesulfonate (**7**). For the S_N2 reaction to proceed, two components are needed: the electrophile and the nucleophile. The arylamine of the amine receptor (**2**) is expected to act as the needed nucleophile over the other amines present because it is more basic than the amines of the urea, and is less substituted than the amine of the cyclen ring.[6] The electrophile needed for this reaction will be formed by converting the hydroxyl of 2-ethoxy ethanol (**6**) into a good leaving group by reaction a with tosylchloride to give 2-ethoxyethyl 4-methylbenzenesulfonate (**7**) as shown in scheme 3.[8] A good leaving group is key to the success of an S_N2 reaction.



(6) **(7)**

Scheme 3. The tosylation of 2-ethoxyethanol (**6**) to produce 2-ethoxyethyl 4-methylbenzenesulfonate (**7**). *i.* The reaction was done with *p*-toluene sulfonyl chloride and pyridine at room temperature for 1.5 hours.

Experimental

Synthesis: Nitro reduction of nitro receptor (1) into the amine receptor (2)

The following method was derived from Pogorelić, et al.[7] and modified by the addition of H_2 gas. The nitro receptor (**2**) (43 mg, 3.68×10^{-2} mmol) was dissolved in methanol (20 mL) by sonication for several hours. Raney nickel slurry (800 μL) suspended in water was added to the solution, followed by sodium borohydride (5.5 mg, 0.147 mmol). Hydrogen gas was put into a balloon, and allowed to leak into the reaction vessel to create a H_2 atmosphere. The reaction mixture was stirred constantly with a magnetic stir bar for 4 h at 35° C in an oil bath. A reflux apparatus was set up to ensure the methanol did not evaporate. The reaction mixture had to be

evaporated under reduced pressure in order to show a clear TLC because of the water present in the mixture. Thin layer chromatography plates obtained of the reaction mixture were stained with I_2 followed by ninhydrin. After the allotted time, the reaction mixture was evaporated under reduced pressure to produce a rusty orange solid. The crude product was dissolved in a 50% methanol/chloroform (MeOH/$CHCl_3$) solution and filtered by gravitational filtration to rid it of any salts present. The crude product was purified by column chromatography using silica gel and a 10% MeOH/$CHCl_3$ solution to give 7.3 μg of product (6.56×10^{-3} μmol). The product yield was 19%. The purified product was dissolved in MeOH-d_4 and subjected to ^{1}H NMR on a 400 MHz Varian AS400 instrument. In addition, the purified product was dissolved in methanol and subjected to time-of-flight (TOF) mass spectrometry.

Synthesis: Tosylation of 2-ethoxyethanol to produce 2-ethoxyethyl 4-methylbenzenesulfonate

The following method was derived from Butler, et al.[8] 2-ethoxyethanol (**6**) (2.9 mL, 0.03 mol) and *p*-toluene sulfonyl chloride (1.9 g, 0.01 mol) were mixed. Pyridine (6.5 mL, 0.08 mol) was then added. The reaction ran for 1.5 h, and it was monitored by TLC using I_2 to stain. After the allotted time the reaction was washed with water to remove any starting material and impurities. The product was a clear, yellow liquid that was more dense than water. Further purification was done by column chromatography with silica and 33% Hexanes/$CHCl_3$. Product yield was 50%. The purified product was dissolved in $CHCl_3$-d_1 and subjected to ^{1}H NMR at 300 MHz to determine the structure.

Synthesis: Attaching 2-ethoxyethyl groups by S_N2 reaction

The following method was derived from Lu et al. (2009).[9] Amine receptor (**2**) (4 mg, 3.60×10^{-3} mmol) and 2-ethoxyethyl *p*-toluenesulfonate (**5**) (3.5 mg, 1.44×10^{-2} mmol) were dissolved in dry dimethylformamide (DMF). Potassium carbonate (K_2CO_3) (2.0 mg, 1.44×10^{-2}

mmol) was added, but it did not fully dissolve. As the reaction proceeded the increase in acidity allowed K_2CO_3 to go into solution. The reaction was stirred constantly with a magnetic stir bar for 72 h at 50° C in an oil bath. The reaction was monitored by TLC using UV-active plates and ninhydrin for staining. The DMF was evaporated under reduced pressure for each TLC sample taken because the DMF would keep the compounds from separating on the TLC plate. The DMF was evaporated under reduced pressure after the allotted time passed. The TLC plates were observed under UV light and then stained with ninhydrin.

Results and Discussion

Research Methodology

The overall goals of this research were to develop a successful synthesis of a water-soluble receptor, the determination of the structure via proton nuclear magnetic resonance (^{1}H NMR) and mass spectrometry, the analysis of the receptor via fluorometric titrations, and the assessment of the utility of this receptor in biomembrane assays. Thin-layer chromatography (TLC) of the reaction mixture was performed for each reaction to determine if a reaction had taken place and to suggest which compound may be the desired product. Ninhydrin was used to stain the plates along with iodine or UV-light to indicate the presence of a primary amine, such as in the amine receptor (**2**), by staining the spot purple and compounds with a secondary amine, such as the ethoxy receptor (**3**), yellow. If TLC provides evidence that the reactions occurred and produced the desired product, the structures of the products were then further determined by ^{1}H NMR spectroscopy and mass spectrometry. If the evidence from the ^{1}H NMR and mass spectra support that the final product is the ethoxy receptor (**3**), fluorometric titrations can determine how well the ethoxy receptor (**3**) quenches the fluorescence of the pyrene derivative dyes. The ethoxy

receptor (**3**) can then be tested on the endovesiculation assay to determine whether the data collected is accurate and precise.

Nitro reduction of nitro receptor (1) into the amine receptor (2)

Several attempts were made to reduce the nitro groups of the nitro receptor (**1**). The first method used sodium dithionite, however, this procedure gave a complex mixture of products as seen by NMR and TLC.[10] The second method used activated zinc and ammonium formate.[11] This method, however, only gave back the starting material. The final method used was derived from Pogorelić, *et al.* and uses a combination of $NaBH_4$ and Raney nickel slurry.[7] This method produced the desired product, but only in small quantities. A small purple spot on the TLC plate revealed with ninhydrin staining indicated a successful reaction.

To improve the reduction using the $NaBH_4$/Raney nickel combination, H_2 gas was added because it works well to reduce less polar groups. To recall, one of the concerns in reducing the nitro group is the possibility of the reduction stopping at one of the intermediates. As the intermediates are formed, they become less polar and the subsequent reduction is very slow, resulting in poor yields of the desired product. By adding hydrogen gas to this reaction, the reaction rate for the reduction of the less polar intermediates increases to an acceptable rate. The total yield of the reaction with the hydrogen gas was 19% after purification. This low yield is most likely a result of a loss of product during purification, incomplete reduction, and reduction of some of the ester groups present. A TLC of a typical reaction mixture is shown in Figure 6. The TLC plate shows that a large purple spot appeared after the plate was stained with ninhydrin. NMR and mass spectrometry confirmed this spot to be the desired product. The other purple spots that appeared at the bottom of the plate might be receptor arms that include partially

reduced arylnitro groups or the esters that were reduced which detached the arm from the cyclen core.

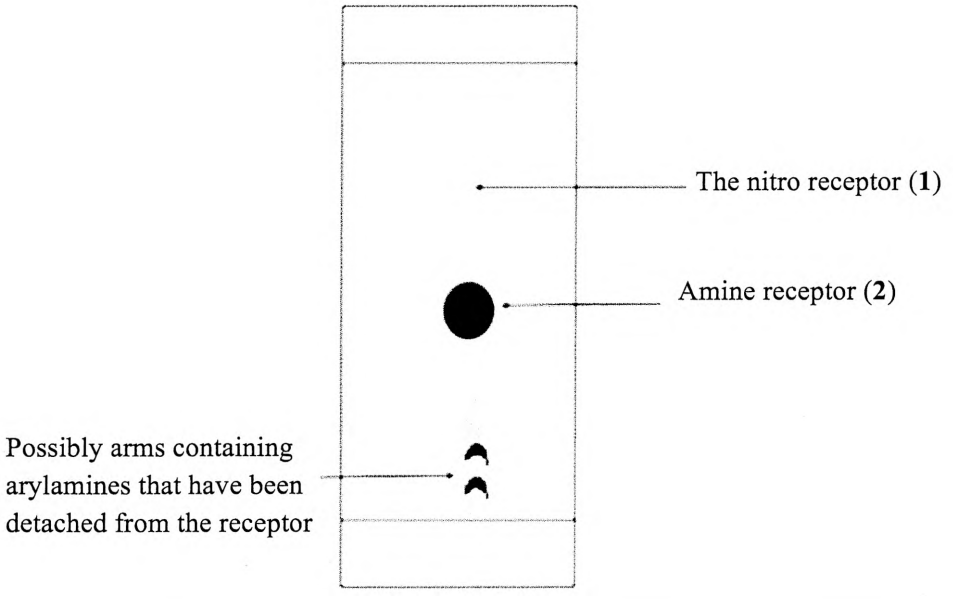


Figure 6. Final TLC plate obtained of the nitro reduction with Raney nickel slurry, $NaBH_4$, and H_2 gas. The nitro receptor (**1**) spot was really small, indicating most of it reacted, but not all of the nitro receptor was converted into the amine receptor (**2**). Any other spots that have not been identified may have been byproducts or the nitro receptor intermediates containing nitroso or hydroxyamine groups. The nitro receptor (**1**) was determined by co-spotting.

Proton nuclear magnetic resonance spectroscopy can be used to elucidate the structure of compounds by indicating the location of the protons in relation to the functional groups and neighboring protons. The ^1H NMR spectrum (refer to Fig. 7) of the nitro receptor (**1**) shows a pair of doublets in the aromatic region for the protons on the benzene ring. The peaks that appear as doublets indicate there is one neighboring proton, and any peaks that appear around 7 to 8 ppm indicate the protons are a part of an aromatic ring such as benzene. Electron-withdrawing functional groups such as nitro groups cause the proton peaks to shift downfield, or the peaks to increase in ppm values. When the arylnitro groups are reduced to arylamine groups, the electron-withdrawing groups are changed into electron-donating groups. The electron-donating groups shift proton peaks upfield, or the peaks decrease in ppm values. The ^1H NMR spectrum of the

possible amino receptor (**2**) produced showed a large shift upfield of about 1 ppm (refer to Fig. 8). This indicates that the functional group on the benzene ring has been converted into an electron-donating group, or the nitro group has been converted into an amine group. The absence of any other doublet pairs indicates that all of the arms of the receptor have the same functional groups and that all of the nitro groups have been reduced completely.

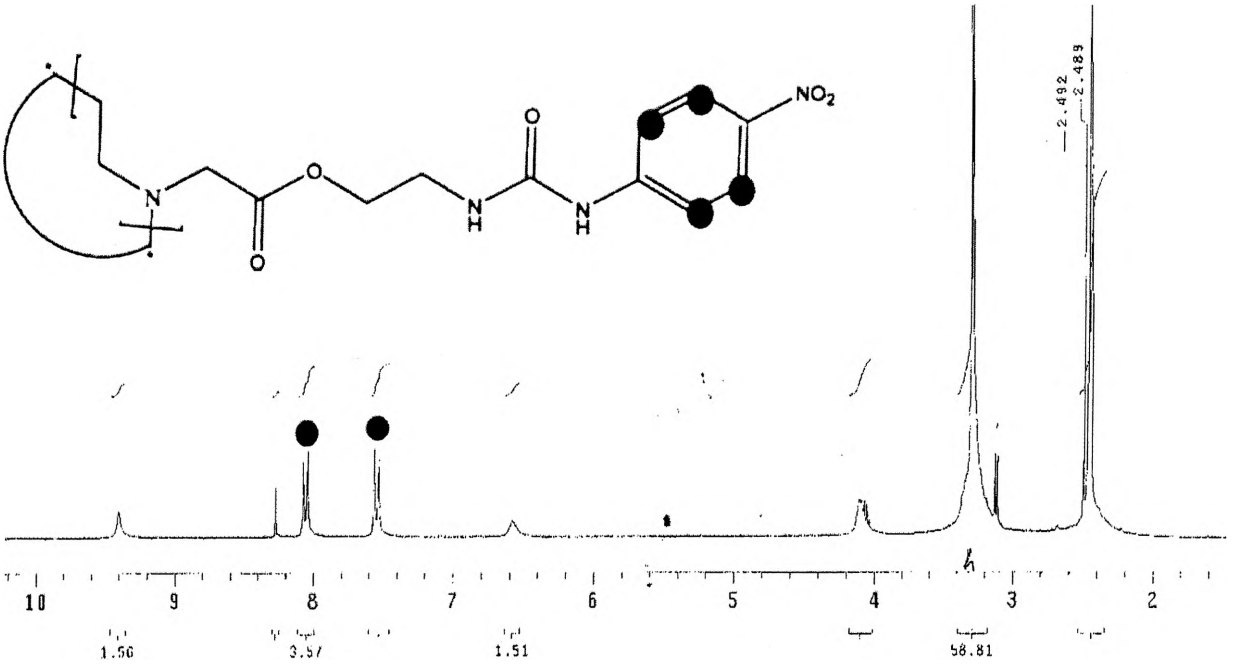


Figure 7. Nitro receptor (**1**) ^{1}H NMR spectrum in DMSO-d_6. The axis is shown in ppm. The aromatic peaks of interest appeared at about 8.05 ppm and 7.55 ppm. The protons associated with each peak are shown by the colored circles.

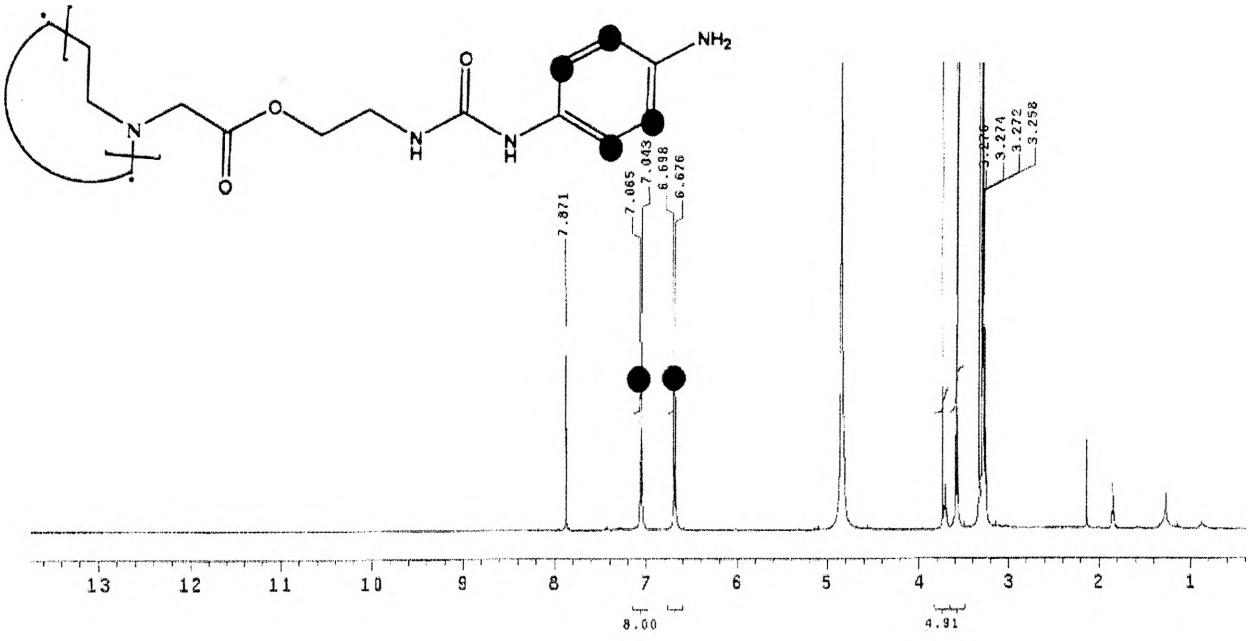


Figure 8. Amine Receptor (**2**) ^{1}H NMR Spectra in MeOH-d_4. The axis is shown in ppm. The shifted aromatic peaks appeared at about 7.05 ppm and 6.85 ppm. The protons associated with each peak are shown by the colored circles.

Mass spectrometry was used to determine if the receptor remained intact during the reduction. As mentioned previously, the reduction of the ester in the nitro receptor (**1**) can result in the arm of the receptor breaking off.[6] If the receptor remained intact the mass spectrometry results would indicate the total mass of the compound to be 1112.19 g/mol. However, the initial mass spectrum revealed no peak with a mass to charge ratio (m/z) close to 1112.19 m/z that would indicate this. Cesium iodide (CsI) was added to the sample in case the amine receptor (**2**) formed a chelate or ionic bond with sodium ions from $NaBH_4$. Sodium ions (Na^+) can suppress the signal on the mass spectrum. Adding CsI to the sample causes some Cs^+ ions to replace Na^+ ions, thus allowing for stronger signal to appear. On the mass spectrum with the sample containing CsI (refer to Fig. 9), the largest mass to charge ratios appeared at 1310.0219, 1311.0559, and 1312.0460 m/z respectively, indicating the presence of a compound with a molecular mass around 1310 to 1312 g/mol. The mass of the receptor, three sodium ions, a cesium ion, and the loss of four protons is 1311.05 g/mol, so the spectrum supports the hypothesis that the compound isolated and is indeed the amine receptor (**2**). The actual amine

receptor (**2**) may have lost four protons found in the urea groups. These protons were replaced by four Na^+, and the addition of CsI allowed one Cs^+ to replace one Na^+.

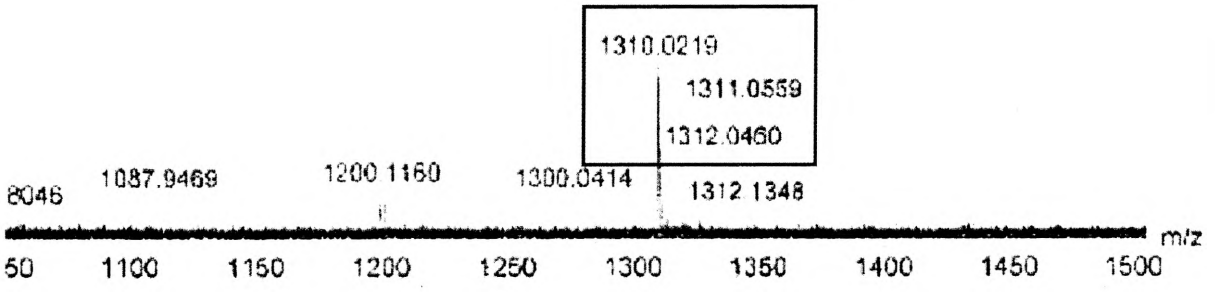


Figure 9. Mass spectrum of the amine receptor (**2**) with CsI. Time-of-flight mass spectroscopy with electrospray was used. Relevant peaks indicating the presence of the amine receptor (**2**) were boxed.

Attaching 2-ethoxyethyl groups by S_N2 reaction

Thin layer chromatography indicated a successful reaction. On the TLC plate shown in Fig. 10, the purple spot of the amine receptor (**2**) and the spot of the 2-ethoxyethyl 4-methylbenzenesulfonate (**7**) decreased slightly as a middle spot increased over time. However, this new spot did not stain yellow from ninhydrin. As mentioned previously, ninhydrin stains a compound yellow in the presence of a secondary amine. No appearance of a yellow color could simply be because the ethoxy receptor (**3**) existed at too low of a concentration to allow the yellow stain to appear. Since the ethoxy receptor (**3**) is less polar than the amine receptor (**2**), the spot above the amine receptor (**2**) can be assumed to be the ethoxy receptor (**3**). There should be a fourth spot indicating the presence of the leaving group, the tosylate. A very faint spot did appear near the baseline of the TLC, but it was hard to distinguish because it was very faint. Since the tosylate is negatively charged, it would appear near the baseline. Therefore, there could be tosylate present in very low concentrations. After 72 hours there was no change in spots, and the reaction appeared to stop. The evidence of low concentrations of the products indicates the possibility of a low yield.

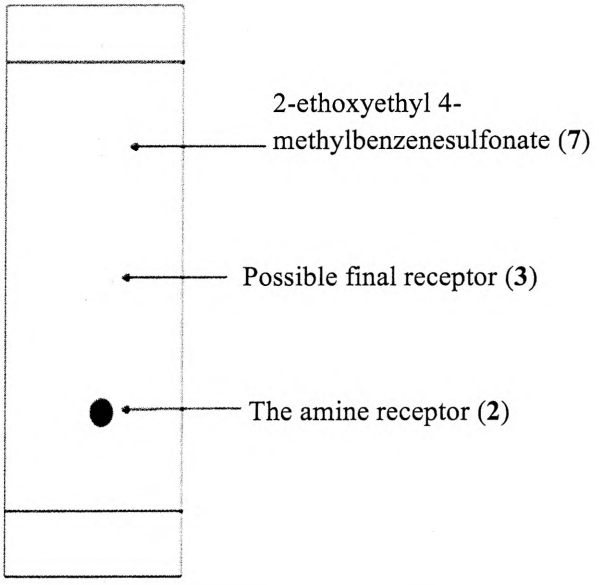


Figure 10. Final TLC plate obtained of the attachment of the 2-ethoxyethyl reaction. A possible spot did appear near the baseline, but it was too faint to make a decent distinction. The middle spot was determined to be the final receptor (**3**) because of its decrease polarity compared to the amine receptor (**2**). The amine receptor (**2**) and **7** were determined by co-spotting.

According to Suresh Narayana (personal communication; a graduate student in the Sidorov research group) the product that formed the middle spot was purified and subjected to analysis by ^{1}H NMR and mass spectrometry to verify its structure.

Follow-up work conducted on the synthesized receptors

After the receptor was successfully synthesized, Narayana performed follow-up work on the receptor. The ethoxy receptor's (**3**) maximum concentration in water was determined. The water-solubility of the ethoxy receptor (**3**) was reported to have a maximum concentration of 0.320 mM. This is an 8-fold increase compared to the original nitro receptor (**1**) which had a maximum concentration of 0.040 mM. The quenching ability of the ethoxy receptor (**3**) and the amine receptor (**2**) were investigated by conducting fluorometric titrations on the receptors with pyranine. The quenching ability of the ethoxy receptor (**3**) and the amine receptor (**2**) were discovered to be very poor when the receptors were applied to the fluorometric titrations.

Therefore, the receptor cannot be utilized in the endovesiculation detection assays or in any other biomembrane assays.

The amine in the receptor may be the cause of the decreased quenching ability because both the ethoxy receptor (**3**) and the amine receptor (**2**) could not quench pyranine effectively. Before the receptor interacts with the dye, intramolecular hydrogen bonding between the amine and the oxygens or nitrogens in the other functional groups would occur. This hydrogen bonding could prevent the receptor from forming a complex with the dye. Intermolecular hydrogen bonding can occur between two separate molecules, as well. To avoid this, current attempts to attach the ethoxy groups to the cyclen core are under investigation.

The results of the mass spectrum of the amine receptor suggest the amine (**2**) and ethoxy (**3**) receptors lack protons from the nitrogens in the urea group. These protons were shown to be necessary in forming the complex with pyrene derivative dyes by hydrogen bonding. Without the protons, the receptors cannot hydrogen bond with the dye, destroying the ability of the receptors to quench the dye's fluorescence.

Acknowledgements

I would like to thank Dr. Vladimir Sidorov and the research group for all of their help and support through this project, the Research Experience for Undergraduates program at Virginia Commonwealth University for allowing me to participate in this program, and the National Science Foundation for funding. I would also like to thank my thesis committee, Dr. Priscilla Gannicott, Dr. Dwight Williams, Dr. Cowden, and Dr. Freier, for their advice and guidance with preparing thesis.

References

1. Winschel, C. A., Kalidindi, A., Zgani, I., Magruder, J. L., & Sidorov, V. (2005). Receptor for anionic pyrene derivatives provides the basis for new biomembrane assays. *Journal of the American Chemical Society, 127*, 14704-14713.
2. Winschel, C. A., Kaushik, V., Abdrakhmanova, G., Aris, S. M., & Sidorov, V. (2007). New noninvasive methodology for real-time monitoring of lipid flip. *Bioconjugate Chemistry, 18*, 1507-1515.
3. Daleke, D. L. (2003). Regulation of transbilayer plasma membrane phospholipid asymetry. *Journal of Lipid Research, 44*, 233-242.
4. Tedesco, M. M., & Matile, S. (1999). Spectroscopic detection of endovesiculation by large unilamellar phosphatidylcholine vesicles: Effects of chlorpromazine, dibucaine, and safingol. *Bioorganic and Medicinal Chemistry, 7*, 1373-1379.
5. Wei, W., Wang, Z., Mizuno, T., Cortez, C., Fu, L., Sirisawad, M., Naumovski, L., Magda, D., & Sessler, J. L. (2006). New polyethyleneglycol-functionalized texaphyrins: Synthesis and in vitro biological studies. *Dalton Transactions,* (16), 1934-1942.
6. Smith, J. G. (2008). *Organic chemistry* (2nd ed.). Boston: McGraw Hill Higher Education.
7. Pogorelića, I., Filipan-Litvića, M., Merkaša, S., Ljubića, G., Cepaneca, I., & Litvić, M. (2007). Rapid, efficient and selective reduction of aromatic nitro compounds with sodium borohydride and Raney nickel. *Journal of Molecular Catalysis A: Chemical, 274*, 202-207.

8. Butler, C. L., Nelson, W. L., Renfrew, A. G., & Cretcher, L. H. (1935). Cinchona alkaloids in pneumonia. I. miscellaneous alkaloids and some hydrocupreine ethers. *Journal of the American Chemical Society, 57*(3), 575-578.
9. Lu, H. H., Lui, H., Wu, W., Wang, X. F., Lui, L. Q., & Xiao, J. X. (2009). Catalytic asymmetric intramolecular hydroarylations of -aryloxy- and arylamino-tethered ,-unsaturated aldehydes. *Chemistry - A European Journal, 15*(12), 2742-2746.
10. Scheuerman, R. A., & Tumelty, D. (2000). The reduction of aromatic nitro groups on solid supports using sodium hydrosulfite (Na2S2O4). *Tetrahedron Letters, 41*(34), 6531-6535.
11. Gowda. (2001). Zinc-catalyzed ammonium formate reductions; rapid and selective reduction of aliphatic and aromatic nitro compounds. *Indian Journal of Chemistry. Section B. Organic Including Medicinal, 40B*(1), 75-79.