Development of New Methods for Liposomal Drug Delivery and the Labeling of Cellular Lipids

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I am submitting herewith a dissertation written by Shahrina Alam entitled "Development of New Methods for Liposomal Drug Delivery and the Labeling of Cellular Lipids." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Chemistry.

Michael D. Best, Major Professor

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Development of New Methods for Liposomal Drug Delivery and the Labeling of Cellular Lipids

A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Shahrina Alam
December 2016
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ABSTRACT

Liposomal drug delivery shows great promise for developing smart delivery materials, especially when a cytotoxic drug needs to be administered. The liposome membrane bilayer structure reduces toxicity through encapsulation, circumventing side effects. Current work in this field is aimed at selectively targeting delivery to diseased cells and controlling the release of encapsulated drugs.

In the first project of this dissertation, we describe the synthesis of a clickable and photocleavable lipid analog for efficient drug delivery to cells and subsequent triggered release. This lipid analog contains a cyclooctyne moiety by which azide-containing drug molecules or fluorophores can be attached using copper-free click chemistry and a photocleavable group for light-driven release. We have validated conjugation of this lipid analog via click chemistry using fluorescence-based liposome derivatization studies and a liposome immobilization microplate assay. Next, fluorescence microscopy was used to show successful delivery of fluorescent dyes to cells and subsequent release. Finally, this lipid was applied for liposomal delivery of camptothecin to cancer cells, which led to a dose-dependent decrease in cell viability.

Metabolic labeling of biomolecules is another topic of interest described in this dissertation. In this project, we sought to design, synthesize and study click-tagged serine analogs that could produce labeled versions of phosphatidylserine in cells to investigate lipid production, trafficking, and to understand the role of
lipids in the instigation of cancer and other diseases. In studies, either Saccharomyces cerevisiae or human cells were treated with these probes, followed by incubation with a clickable fluorescent reagent and confocal fluorescence microscopy to detect labeled products. To determine the labeled lipids, the extracted cell samples were subjected to tandem mass spectrophotometry and a number of phospholipids were detected. Lastly, we also showed that click labeling of PS, a lipid upregulated on the outer leaflet of cancer cell membranes, can be utilized as a tool for delivery the delivery of liposomes bearing partner clickable tags, which has been confirmed via a confocal microscope. Finally, we have synthesized a number of L-serine analogs as potential PS synthase inhibitors and worked to evaluate the efficacy of these compounds using a PS synthase assay.
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<table>
<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA</td>
<td>acyl-coenzyme A</td>
</tr>
<tr>
<td>ADIBO</td>
<td>azodibenzocyclooctyne</td>
</tr>
<tr>
<td>(Boc)₂O</td>
<td>di-tert-butyl dicarbonate</td>
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<tr>
<td>Candida spp.</td>
<td>Candida species</td>
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<tr>
<td>CH₂Cl₂</td>
<td>dichloromethane</td>
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<td>CHCl₃</td>
<td>chloroform</td>
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<td>CDP-DAG</td>
<td>cytidine diphosphate-diacylglycerol</td>
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<td>CMP</td>
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<tr>
<td>CPT</td>
<td>camptothecin</td>
</tr>
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<td>CUAAC</td>
<td>copper catalyzed azide-alkyne cycloaddition</td>
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<tr>
<td>DAG</td>
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<td>DCC</td>
<td>dicyclohexylurea</td>
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<td>DDQ</td>
<td>2,3-dichloro-5,6-dicyanohydroquinone</td>
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<td>DIEA</td>
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<tr>
<td>DIFO</td>
<td>difluorinated cyclooctyne</td>
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<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
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<td>DMF</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DOPC</td>
<td>Dioleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>Dioleoyl phosphatidylethanolamine</td>
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<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>Et&lt;sub&gt;3&lt;/sub&gt;N</td>
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<td>Infrared</td>
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<td>K&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt;</td>
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<tr>
<td>LPA</td>
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</tr>
<tr>
<td>LPC</td>
<td>Lyso-phosphatidylcholine</td>
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<td>LPS</td>
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<td>LPGs</td>
<td>Light protecting groups</td>
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<td>m</td>
<td>Meta</td>
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<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Sodium carbonate</td>
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<td>Sodium hydroxide</td>
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<tr>
<td>o</td>
<td>Ortho</td>
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<tr>
<td>o-NB</td>
<td>Ortho-nitrobenzyl</td>
</tr>
<tr>
<td>p</td>
<td>Para</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pHLIP</td>
<td>pH low insertion peptide</td>
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<tr>
<td>PIP</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PIP₅K</td>
<td>phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PIPₙn</td>
<td>phosphatidylinositol polyphosphate</td>
</tr>
<tr>
<td>PMBCl</td>
<td>para-methoxybenzyl chloride</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SSNs</td>
<td>stimuli sensitive nanoparticles</td>
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<td>sPLA2</td>
<td>secretory phospholipase A2</td>
</tr>
<tr>
<td>SPAAC</td>
<td>strain promoted azide-alkyne cycloaddtion</td>
</tr>
<tr>
<td>TMSBr</td>
<td>trimethylsilyl bromide</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TMP</td>
<td>tetramethylpiperidine</td>
</tr>
<tr>
<td>TsCl</td>
<td>p-toluenesulfonyl chloride</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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CHAPTER ONE
INTRODUCTION TO DRUG CARRIER VESICLES AND
TECHNIQUES FOR EFFICIENT DRUG DELIVERY

1.1 Common Drug Delivery Vehicles

Modern research in drug delivery and release involves the development of smart biotunable drug carriers, which may involve the use of targeting groups for delivery to specific diseased sites and/or a stimulus-driven release technique that ensures discharge of payloads in the biological system. Pharmaceutical research has been continued in search of ideal drug carriers with selectivity for delivering drugs to diseased cells that are often toxic to healthy tissues. Furthermore, if the drug is unencapsulated or unconjugated to a delivery vehicle, a number of complications such as poor localization, off-site effects, and short circulation periods^1^ diminish the overall efficacy of the drug. For example, well-known drug carriers including micelles, cyclodextrin, nanotubes, dendrimers (Figure 1.1) etc. are insufficient to overcome many of these obstacles. The reasons for this include the hydrophobic nature of these carriers, their ability to encapsulate only hydrophobic molecules, short circulation periods in the blood stream and to the lack of biotunable properties. Carbon nanotubes are highly hydrophobic polycyclic structures that have high surface areas for attachment of drugs, proteins, or other molecules,^2^ whereas micelles are only capable of hydrophobic entrapment. Dendrimers consist of polymeric materials with a high amount of branching resulting in large surface areas for functionalization, whereas a
nanoshell plasmons are spherical nanoparticles consisting of a dielectric core that is covered by a thin metallic shell.

In 1960, a new idea for the delivery vehicle was established by Bingham who came up with the concept of liposomes; spherical bilayer structures formed by lipids when they are suspended in aqueous medium. Liposomes can be widely applied to carry polar drugs in the internal aqueous environment and the hydrophobic membrane core can be utilized to entrap nonpolar drugs. One of the most beneficial aspects of liposomes is that the membrane bilayer structure reduces toxicity by encapsulating the cytotoxic chemical, which is maximized when the drug is selectively released at the location of the diseased tissue.

![Diagram of common drug delivery vehicles](image)

**Figure 1.1:** Common drug delivery vehicles suitable for entrapping drug moieties.
Moreover, liposomes are biocompatible as they are mimics of natural lipids and cell membranes, thus diminishing clearance by the immune system. Liposomes including DaunoXome (danorubicin) and Myocet (doxorubicin)\(^3\) showcase this low toxicity and are already available in the market as drug carriers.\(^4\) Still these popular drugs are facing challenges since they are cleared by the reticuloendothelial system (RES) in a short period of time. Incorporation of polyethylene glycol (PEG) onto the surfaces of liposomes during the formulation process has been used to enhance the circulation time as well as the overall efficiency of drug delivery.

### 1.2 Derivatization of Liposomal Surfaces

The derivatization of lipid-based nanodrug carriers enables numerous therapeutic applications and can be performed either before or after self-assembly. The ability to functionalize liposomes of varying composition with different targeting groups makes these carriers beneficial as biotunable systems compared to other candidates including micelles, albumin, nanodrugs, and lipid-coated nanoparticles. Furthermore, designing a nanodrug carrier aimed for highly specific delivery to the surfaces of targeted cells is crucial. This results in highly tunable biocompatible medicinal nanocarriers that have been established as effective tools for imaging and therapeutic applications.\(^5\)\(^6\)\(^7\)\(^8\)

More recent strategies for the post-conjugation of liposomal membranes include click/bioorthogonal chemistry including the copper-catalyzed azide-alkyne cycloaddition (CuAAC), copper-free click chemistry, the Staudinger ligation,
tetrazine/trans-cyclooctene inverse electron demand Diels-Alder (IEDDA) cycloaddition as well as enzymatic modification and the His-Tag chelation strategy.\textsuperscript{17} Maleimide/thiol chemistry, prior to click chemistry, has been used for derivatization of intact liposomes. An alternative method is post-modification—here 5-10 mol\% of synthetic lipids with short PEG-linked head groups with a wide variety of functional groups such as amines, carboxylic acids, thiols or maleimides—can be self-assembled into the bilayer. PEGs can then be grafted on after liposome formation via the appropriate chemistry, be it amide conjugation, hydrazine bonding, thioester formation or disulfide bond formation\textsuperscript{9}.

Later, click chemistry emerged as a class of high-yielding reactions that is broadly tolerant to conditions and other functional groups that are present.\textsuperscript{10} As a result, click reactions also benefit from their bioorthogonal nature, meaning that the reactive partner functional groups used for these reactions can selectively react with one another in high yield without side products within the complex environment of biological systems.\textsuperscript{11-13} Since its inception, the click chemistry philosophy has been advantageous for numerous applications including liposomal drug delivery.

1.2.1 Azide-Alkyne Cycloaddition: The azide-alkyne cycloaddition reaction, the most prominent member of a family of reactions known as “click chemistry”, involves a 1,3-dipolar cycloaddition reaction forming a triazole ring. This reaction was first reported by Huisgen\textsuperscript{14} and later in 2002, modified by Sharpless and co-workers\textsuperscript{15} and Meldal and co-workers.\textsuperscript{16} This modification introduced the use of a
copper (I) catalyst that dramatically improved overall reaction rate and yields. Moreover, other benefits of the copper-catalyzed azide-alkyne cycloaddition include improved chemo- and regioselectivity, reaction scope, and water compatibility. For instance, the proximity between the alkyne and azide reactive partners caused by reporter binding increases the reaction rate and has been used for templated synthesis of high-affinity enzyme inhibitors. Lastly, as with other cycloadditions, the reaction rate is enhanced when the reactive groups are polarized, and thus, it is common to include an electron withdrawing or donating group proximal to the alkyne. Moreover, several studies have been performed to elucidate the reaction mechanism and optimize reaction conditions and it has been found that the addition of a ligand that chelates copper enhances the reaction rate and stabilizes the metal.

1.2.2 Copper-Free Azide-Alkyne Cycloaddition: Although the copper-catalyzed azide-alkyne cycloaddition is effective for *in vitro* biological studies, the mandatory application of the copper catalyst can be detrimental to *in vivo* studies. The need for the copper in this click reaction requires the delivery of this catalyst to the reaction site, which is problematic since copper is toxic to living cells. The strain promoted azide-alkyne cycloaddition reaction (SPAAC) (also known as the copper-free azide-alkyne cycloaddition), reported by Bertozzi and co-workers, overcomes this obstacle. This reaction is effective in the absence of copper catalyst due to the incorporation of the alkyne within a strained cyclooctyne ring, which increases its intrinsic reactivity.
Figure 1.2: Bioorthogonal reactions that are effective for bioconjugation both *in vitro* and *in vivo*.

A variety of cyclooctyne reagents have been developed for copper-free click chemistry. For example, difluorocyclooctyne (DIFO, **Figure 1.2**) reagents led to enhanced reactivity due to the polarization caused by the electron withdrawing fluorine groups.\textsuperscript{24} Copper-free click reactions have proven to be invaluable for in vivo labeling.\textsuperscript{25-27} For example, Baskin *et al.* demonstrated selective labeling of biomolecules such as glycans employing a Cu-free variant of click chemistry in living systems.\textsuperscript{28} The enhanced reactivity of DIFO was important for enhancing
bioorthogonal imaging of azide-tagged sugars in living organisms. Additionally, Ning and co-workers synthesized a dibenzocyclooctyne reactant with increased hydrophilicity.29, 30 Specifically, azodibenzocyclooctyne reagents, commonly known as ADIBO30, 31 or DIBACO have become very popular in bioorthogonal chemistry for their wide applications in vivo studies.27, 32 These compounds participate in a copper free click reaction at a faster reaction rate. Recently, near infrared (NIR) fluorogenic probes activated by bioorthogonal chemistry have become attractive for visualization of biomolecules in living systems.46 Here labeled cyclooctyne species can be clicked via SPAAC onto NIR fluorogenic azide probes and later monitored on microscopy at near IR range, where tissue penetrance is highest and background fluorescence from the excess probe and endogenous biomolecules is minimized at these wavelengths.

1.2.3 Staudinger Ligation: The Staudinger ligation reaction is another example of a biorthogonal reaction suitable for in vivo labeling. This reaction emerged as a modification to the Staudinger Reduction reaction33 by the formation of an azaylide intermediate as a result of an intramolecular attack of an electrophile, commonly to an ester, attached to a phosphine reagent. While the original reaction product contained a phosphine oxide moiety, a later modification termed the “traceless Staudinger ligation” was designed such that the phosphine is released, resulting in a simple amide bond linkage.34, 35 The original reaction has been employed for aza-ylide capture using several phosphine reagents containing different groups as shown in Figure 1.2. Since this time, the
mechanism of the reaction and other details have been probed in depth\textsuperscript{36}, and a number of replications have been reported, such as fluorogenic versions\textsuperscript{37}, including an FRET-based system\textsuperscript{38}, as well as reagents with increased water solubility\textsuperscript{39}.

1.2.4 Applications of Bioorthogonal Chemistry in Functionalization of Membranes

The copper-catalyzed azide–alkyne cycloaddition (CuAAC) has been effectively employed to derivatize membrane surfaces.\textsuperscript{40, 41} This was initially accomplished for in-situ modification of liposomes, in which a lipid analog bearing a terminal alkyne group was used for chemoselective conjugation of molecules containing an azido group. The application of this efficient conjugation method has been extended for the attachment of ligands for liposome targeting,\textsuperscript{42} and decoration with DNA to enable the study of DNA-mediated bilayer fusion and docking.\textsuperscript{43} Recently, in 2014, Smyth and co-workers described a protocol for functionalization of exosomes with alkyne groups. Exosomes are membrane vesicles 30–100 nm in diameter carrying blood, urine, and saliva, present in almost all cells. They participate in cell-to-cell communication and can be engineered to deliver protein, mRNA, and miRNA cargo molecules to recipient cells \textit{in vitro} and \textit{in vivo}. Here, they first successfully modified the exosomes with alkyne groups and next employed copper catalyzed azide-alkyne cycloaddition (click chemistry) for bioconjugation of small molecules and macromolecules to the surface of the exosomes.\textsuperscript{44}
On the other hand, copper-free click chemistry using a strained cyclooctyne moiety offers advantages since it does not require any added metal catalyst. For instance, Bostic and co-workers first reported membrane functionalization using copper free click chemistry employing azido tagged synthetic lipid.\textsuperscript{45} Blenke \textit{et al} successfully conjugated a small molecule azide with liposomes, and a number of ligands were coupled per liposome at a reasonable reaction rate.\textsuperscript{46} They demonstrated the conjugation of a larger azide-modified protein and the number of protein ligands coupled was found to be sufficient for liposome targeting to cells. Essentially, their study revealed the site-specific conjugation of protein ligands to liposomal surfaces via SPAAC.

Finally, the Staudinger ligation has found numerous applications for bioconjugation since it is a high yielding reaction at room temperature in an aqueous medium with high specificity. Zhang \textit{et al} developed liposomal surface functionalization through the Staudinger ligation reaction employing lactose as a model carbohydrate.\textsuperscript{47} Here, the reaction conditions did not alter the integrity of the bilayers in terms of liposome size and leakiness and provided perfectly functional vesicles. The reaction was performed under mild conditions in aqueous buffers without a catalyst, in high yields and with reasonable reaction times. For this, they synthesized an unprotected lactosyl derivative carrying an ethyl spacer functionalized with an azide group followed by derivatization to generate glycosylated liposomes. Consequently, the Staudinger ligation reaction between liposomes bearing synthetic anchor lipids with terminal triphosphine and
the azido group on lactosyl analogs enabled the liposome decoration. This flexible approach can be widely used for the ligation of water soluble molecules, can tolerate many chemical functional groups, and is expected to be convenient for the coupling of other potential ligands to liposomes.

1.3 Passive Targeting Using Stealth Liposomes

Passive targeting of liposomes during drug delivery is driven by the inherent selectivity of liposomes for entering tumor cells, which is a product of the enhanced permeability and retention (EPR) effect. Normal capillary gaps around endothelial cells are 5-10 nm,\textsuperscript{48} while carcinomas and other neoplastic or inflamed tissues have a much larger gap area. Maeda et al. recently explained biological aspects of how and why the blood vessels around cancer cells have an increased porosity that allows liposomes up to 400 nm or greater to accumulate around the tumorous and inflamed tissue.\textsuperscript{49} In addition, recent studies have demonstrated how biocompatibility, surface charge, and circulation time can be utilized to magnify the EPR effect in targeting diseased cells with liposomes. Initial studies have suggested that a neutral to a slightly cationic charge of the liposomal surface is optimal for exploiting the EPR effect, but more in-depth research is required.\textsuperscript{6}

The enlarged capillary gaps surrounding tumors and inflammation sites\textsuperscript{50} associated with EPR can be accessed by nanoparticles around 100 nm in diameter, which are considered as ideal. Nevertheless, these liposomes will be recognized by opsonins, a class of serum proteins that non-covalently bind to the
surfaces of larger nanoparticles, marking them for phagocytosis beforehand and clearance via the reticuloendothelial system (RES), also referred to as the mononuclear phagocytic system (MPS), unless carriers contain any camouflage that can circumvent the body’s immune responses. Shielding nanoparticles from opsonins is one good way to increase the circulation of liposomal drugs. This concept eventually led to the idea of shielded (aka stealth) liposomes that use PEG chains, which are chemically introduced at the surface of the nanoparticle to discourage binding of opsonins, thereby opposing the ability of the RES to remove the nanoparticles from the blood stream. It is noteworthy that some research has suggested that the increase in circulation time in PEGylated liposomes is not related to opsonin interference, but rather due to simply decreased liposomal aggregation, a known feature of PEGylated vesicles. Moreover, increasing circulation time allows for the EPR effect to recruit liposomal drugs to angiogenic sites but it also enhances drug exposure to other healthy tissues.

Figure 1.3 describes two popular PEG coatings: the brush mode and mushroom-like globular PEG coating. Li and co-workers demonstrated a formulation for brush mode with >8 mol% PEG that is ideal for total protection from opsonization even though previous studies have shown that PEGylation above 9 mol% will result in lateral repulsion of the brush-like PEG morphology, destabilizing the membrane. Noble et al. reported a formulation for a mushroom configuration where 5-9 mol% of PEG(2000) is utilized that completely masks the
vesicles from opsonins, escaping the RES and allowing for liposomal accumulation. PEGylated liposomes and other nanoparticles are commonly formed by incorporating PEG-lipids or PEG-polymers and formed via self-assembly. Many PEGylated lipids, including those with different lengths of PEG-chains (i.e. PEG(2000) and PEG(5000)) connected to lipid anchors such as PE are commercially available. This is true for many nanoparticles as well. PEG groups can be post-inserted, as long as the appropriate functional group is expressed on the outer membrane after self-assembly.

Figure 1.3: Two popular PEG morphology used on liposomal membranes: high density ‘brush’ mode (left) and low density globular ‘mushroom’ mode (right).

It is important to note that these two strategies (self-assembled modification and post-modification/post-insertion via a synthetic handle) are the primary means by which liposomal surfaces are functionalized for targeting and cargo release as well. Although PEGylated liposomes stealthily evade the immune
system, eventually they accumulate in the tumor interstitial space as a result of the EPR effect. PEG coatings inhibit liposome-liposome membrane aggregation, which is beneficial for opsonin evasion and increases circulation time of liposomal nanodrugs. Nevertheless, for the same reason, PEG coatings inhibit endocytosis and confirm delivery of liposomal payloads when PEGylated nanodrugs reach the tumor interstitial space.

1.4 Directed Delivery using Targeting Groups

In delivering drugs to cancer cells, generally, proteins that are overexpressed on the cell surface due to disease onset are targeted to achieve selectivity. Very often it has been noticed that cancer cells are enriched in certain protein classes, receptor proteins of similar types are overexpressed concurrently in one strain of cancer or there is variably among different types. For example, αβ integrins are one novel class of transmembrane glycoproteins overexpressed in tumors that can be exploited as a stimulus for targeted nanodrugs. The α5β1 receptor is also found to be overexpressed in breast cancers and can be targeted. For this, generally, a fibronectin-mimetic peptide is incorporated into liposomal drugs to decorate the nanodrug surface membrane and deliver it to cells, providing a promising therapeutic strategy. This protocol exhibits minimum cytotoxicity comparable to that of the free drug in vitro and can be considered a promising sign when evaluating the clinical potential of nanodrugs. Cell surface receptors are targeted by a number of commonly used antibodies.
Estrogens, antibodies against progesterone and HER2 have been incorporated into liposomal drug formulations to selectively target breast cancer tissues. For targeting ovarian cancer cells where epidermal growth factor receptors (EGFRs) are overexpressed, EFGR-targeted monoclonal antibodies are doped into liposomal formulations. Canonical estrogen receptor (ER) is another example of an over-expressed protein in breast cancer. To target the ER effectively, drug-loaded liposomes are formulated with ER antagonist tamoxifen into the membrane. Nagase et al. and co-workers formulated liposomes with the lipophilic cancer-preventative tamoxifen and encapsulated doxorubicin using an ammonium sulfate gradient. This formulation of stealth nanoparticles displayed promising results since these liposomes can encapsulate tamoxifen and doxorubicin to decrease the dosage of doxorubicin needed and also actively target the drug to breast cancer. Moreover, estrone is another promising targeting moiety for ER, and liposomal surfaces can be decorated with estrone via conjugation to PEG linkers. Using this PEG-linked targeting strategy, accumulation of doxorubicin-loaded liposomes was 24 times higher than the free drug using this PEG-linked targeting strategy. Another antibody, anti-CXCR, was associated with drug-loaded liposomes using dioleoyl phosphoethanolamine-\(N\)-dodecanoyl (N-dod-PE) to target a specific chemokine receptor (CXCR4).56

1.5 Drug Releasing Techniques

Stimuli sensitive nanoparticles (SSNs) are engineered to change in composition or conformation upon exposure to cellular/extracellular or
intrinsic/extrinsic stimuli which trigger the release of contents selectively at diseased tissues. This change in composition/conformation brings about alterations in the physicochemical and/or drug release properties of these systems. Thus, SSNs demonstrate a “load-and-release” effect either due to attached modalities or to the components themselves. The key benefit of using lipids, polymers and macromolecules is that they intrinsically possess the dynamic properties that are favorable for generating SSN composite materials. Thus, it is crucial to have SSNs bearing sharp physical and chemical sensitivities to instigate release of associated cargo upon a subtle change in surrounding conditions. These physical and chemical properties of SSNs greatly influence the complex physicochemical properties of membranes such as bilayer stability, phase separation, and fluidity in suitable environmental circumstances. Once the nanocarriers enter their desired site of action and are exposed to the specific stimulus that they are tuned for, the bioactive cargo is freed in a manner dependent on its method of inclusion. Entrapped bioactive molecules are commonly released via structural changes in the carrier architecture (i.e. cleavage of the shell, charging of functional groups or carrier degradation). Bioactive payloads can also be directly conjugated to the nanocarrier, for which breakage of the linker between the bioactive molecule and the carrier is the mechanism of triggered release in this instance. Stimuli sensitivity either exists inherently in the nanocarrier building blocks or can be imparted chemically. The unique biochemistry of diseased cells, such as cancers, helps to enhance the
responsive effects of SSNs and can eventually lead to precise temporal and spatial drug delivery, improving therapeutic efficacy with diminished off-target effects.

1.5.1 Internal Stimuli Triggered Release

Stimuli-sensitive drug delivery, or smart delivery, is an extension of the concept of active targeting that advances the goal of delivering the cargo at the diseased site and over the desired time frame. Stimuli-sensitive drug delivery or smart delivery systems use a local stimulus or pathological change occurring in the targeted tissue as a trigger for release. For example, lower pH and higher temperatures are very common traits of tumors and other inflamed tissues compared to most healthy tissues. These factors are termed intrinsic stimuli (Figure 1.4) since they result from conditions characteristic of diseased tissues. Furthermore, greater redox potential is generally observed in cancerous tissues due to altered enzyme expression, which may be present in small quantities or may not be present at all in normal tissues. However, the differences in these intrinsic properties of the diseased cells only vary slightly from ideal physiological conditions. For example, the average extracellular pH of cancer cells has been measured as 6.5–6.9 compared to 7.2–7.4 for normal tissue. To release drug species from PEGylated liposomes, this property of cancer cells is generally exploited. There are cleavable PEG moieties that can be removed from the membrane to allow for endocytosis. For example, liposomes with pH-sensitive diortho ester bonds by which PEGs are linked or thiol-conjugated PEGs will
clease their PEG camouflage in acidic environments\textsuperscript{59}, including the vicinity of tumors. Other modified PEG polymers will cleave in acidic environments as well, such as polyglutamic acid–graft-methoxy polyethylene glycol (PGA–g-PEG).\textsuperscript{60} The biodegradable disulfide bond can also be used to imbue liposomes with shreddable PEG coatings.\textsuperscript{61}

Figure 1.4: Drug releasing techniques utilizing external or internal stimuli to release entrapped drug at desired site.

1.5.2 External Stimuli Triggered Release

On the other hand, external stimuli can be employed to trigger release in the region of interest such as by applying local heat to induce hyperthermia or ultrasound or near infrared light to bring about local delivery of cargo. This approach can be advantageous in that it is not dependent on the properties of
the targeted cells and can be administered in a more localized manner, although the delivery of stimuli to the desired location can be challenging.\textsuperscript{62}

1.6 Light-Induced Releasing Technique

Photo-induced alterations in the properties of lipid carriers have become a popular approach for triggered release of contents. These systems commonly involve a self-assembled system that undergoes a change in structure upon photocleavage or cleavage of a linker by which a drug is covalently attached to the nanocarrier.\textsuperscript{63-65} Photo-induced delivery applications benefit from the capability to apply irradiation in a temporally and spatially controlled manner using a remote source. However, one of the shortcomings of these light triggered releasing techniques with one-photon photocleavable chromophore is that the ultraviolet (UV) wavelength range employed for excitation discourages their applicability since many biomolecules absorb irradiation in this range, leading to their photodecomposition as well as the inability of the beam to penetrate tissue. Therefore, it is advantageous to develop photoinitiated systems that are activated in the near-IR (NIR) range, which benefit from enhanced penetration and decreased damage to cells.

The properties of light that can be easily regulated render irradiation as a suitable means of release. These properties include timed release of lipid carriers at desired sites, control over properties such as wavelength, intensity, duration, and localization.\textsuperscript{62} Light is used for many applications, including crosslinking in protein–lipid binding and photoaffinity labeling.\textsuperscript{66} It can also be utilized for the
activation or deactivation of certain molecules by decomposing photochemically-responsive moieties. For this reason, light protecting groups (LPGs) have been synthesized. One of the more studied LPGs is the 2-nitrobenzyl group. Other groups include 7-nitroindoline, coumarin-4-ylmethyl, \( p \)-hydroxyphenacyl, and nitrophenethyl (Figure 1.5).  

![Figure 1.5: Examples of photocaging groups which aid in light triggering release of payloads from drug delivery vehicles.](image)

Recently, Dong and co-workers utilized a photocleavable coumarin chromophore to synthesize a novel two photon-sensitive amphiphile with two alkynyl groups as a two-photon-cleavable segment (Figure 1.6). This design of stable assemblies of surfactants was found to exhibit potential for dose-controlled triggered release in biomedical applications. The main advantage is that either UV light (365 nm) or a NIR pulse laser (800 nm) can be applied for photolysis of the coumarin units to expose the cargo. The 2-nitrobenzyl photocleavable group has also been widely applied to construct light-triggered switchable lipid systems.
Figure 1.6: Examples of lipid analogs developed for photochemically induced drug release, including 0-nitrobenzyl in NVOC-DOPE (1.1), dithianes (1.2) and coumarin (1.3).
For example, Zhang et al. synthesized a novel lipid, 6-nitroveratryloxycarbonyl-PE, that is a caged derivative of dioleoyl phosphatidylethanolamine (DOPE), for assembling bionanocarriers.\textsuperscript{70} DOPE is a non-bilayer lipid at physiological pH that adopts inverse cubic and inverse hexagonal structures. On the other hand, \textit{N}-acylated PEs are negatively charged phospholipids and can form kinetically stable liposomes. Photolysis of NVOC-DOPE (1.1) destabilizes the lipid bilayer, which eventually leads to liposome disruption and release of encapsulated payloads. This is a promising photochemically triggered delivery system to initiate a vast range of dynamic membrane processes, such as membrane fusion and the activity of enzymes embedded within membrane bilayers. However, the UV light requirement to induce photocleavage of lipids is a technical drawback, limiting the ability to monitor the subsequent membrane process. Two-photon chromophores are a promising solution since excitation using IR wavelengths is less detrimental to biological systems and is more applicable for biomedical delivery protocols. Nagasaki \textit{et al.} synthesized a cationic lipid bearing an \textit{o}-nitrobenzyl moiety as a photocleavable spacer between its hydrophobic and hydrophilic regions, where the polar hydrophilic sections constituted of either the amino acid residue lysine or arginine.\textsuperscript{71} They examined the transfection efficiency of resulting photoresponsive lipoplexes on COS-1 cells and found enhanced transfection as a result of photochemical decomposition of lipids compared to transfection without irradiation.
1.7 Importance of Lipids in Cell Membranes

Cell membranes not only separate the interior of the cell from the extracellular space, but also form the boundary between the internal compartments of eukaryotic cells, including the nucleus and cytoplasmic organelles. Cell membranes are crucial for maintaining the rigid structure of the cell and are responsible for multifaceted functions that regulate cell activities. All cell membranes are comprised of a common structural organization: phospholipid bilayers with associated proteins. These membrane proteins play important roles in many specialized functions; some act as receptors that allow the cell to respond to external signals, some are responsible for the selective transport of molecules across the membrane, and others participate in electron transport and oxidative phosphorylation. On the other hand, phospholipid compositions in cell membranes vary among different compartments depending on lipid metabolism and trafficking. Lipid molecules with two hydrophobic fatty acid chains linked to a phosphate-containing hydrophilic head group are classified into two categories based on their functions in membranes: bulk lipids and signaling lipids. As the name implies bulk lipids represent the primary component of cell membranes, whereas signaling lipids present in much smaller percentages serve as functional molecules and site-specific ligands. These signaling lipids include phosphatidic acid (PA), diacylglycerol (DAG) and the family of phosphatidylinositol polyphosphates (PIPₙS). These lipids have been actively explored and
investigated to understand their roles in crucial processes such as membrane-protein interactions.

Protein-membrane binding interactions represent key molecular recognition events that control vital cellular processes including signal transduction events, viral cell entry as well as other processes, and aberrations in binding interactions trigger several diseases.\(^7^2\) For instance, fluctuations in DAG have been linked to the onset of cancer\(^7^3-^7^5\) and diabetes,\(^7^5\) etc. Moreover, phosphatidylinositol 3-kinase (PI-3K) converts PI-(4,5)-P\(_2\) to PIP\(_3\), the latter of which binds and activates Akt, finally activating several events that regulate the cell cycle. The reverse conversion is promoted by PTEN. Consequently, enzymes that interconvert PI-(4,5)-P\(_2\) and PIP\(_3\) are among the most commonly mutated proteins in cancer.\(^7^6\) Furthermore, it has been determined that phosphatidylserine (PS), normally present at the inner leaflet of the plasma membrane, is translocated into the outer leaflet during apoptosis and the onset of many cancers. This results in PS acting as a biomarker that could be exploited for the selective detection or targeting of cancer cells. Due to the key roles that lipids play in cancer and other diseases, it is necessary to characterize binding activities to fully understand their biological complications.

### 1.8 Metabolic Labeling by Bioorthogonal Reporters

A growing area of chemical biology strives to probe biomolecules in living systems by using bioorthogonal chemical reactions (that is, reactions that do not interfere with biological processes). Such reactions must have fast rates under
physiological conditions with high specificity in the presence of surplus complex functional groups. Metabolic labeling refers to methods in which the endogenous biosynthesis and modification machinery of living cells is used to convert substrate analogs into labeled versions of natural biomolecules. Often, the tag that is incorporated into the substrate derivative is a clickable group, which enables their selective modification with fluorescent dyes or affinity reagents for analysis. This facilitates the detection of the localization and function of biomolecules within cells, which is challenging because of the vast complexity of metabolites present in cellular systems. However, labeling with genetically encoded reporters frequently is incapable of monitoring many biomolecules, such as nucleic acids, lipids, and glycans, as well as various posttranslational modifications. Here, labeling these biomolecules in living systems employing biorothogonal chemical reactions has become appealing and expedient. The biororthogonal chemical reporter strategy involves the incorporation of unique functionality into targets of interest, followed by chemical labeling with a small-molecule probe (Figure 1.7). Ideally, the substrate analog containing a chemical reporter (green circle, Figure 1.7), in this case an azido group, is fed to cells for incorporation into products through normal metabolic pathways. This is accomplished by appending the reporter to substrates that can be used by the cell's own metabolic machinery. Next, these labeled target products are detected using a bioorthogonal reaction.
Figure 1.7: Strategy for metabolic labeling of biomolecules in living systems. A chemical tag (i.e. azide) linked to a substrate (green circle shape) is incubated with cells to produce labeled products through normal cellular metabolism. Next, the appended tag can be modified via bioorthogonal reaction with an exogenously delivered reagent (brown asterisk). This allows for the selective labeling and detection of tagged products.
A common strategy for post-labeling involves click chemistry since the azide and alkyne moieties are small and thus minimally perturb the natural function and modification of probes. For example, amino acids bearing bioorthogonal functional groups can be accepted by the translational machinery of a cell and incorporated into proteins.\textsuperscript{78, 79} Here, labeling of proteins using radiolabeled amino acids and in cell cultures (prokaryotic or eukaryotic) has been popular. This technique results in a measurable incorporation of radioisotopes in proteins, which can be easily detected by scintillation counting or mass spectrophotometry.\textsuperscript{80} Furthermore it has been reported that cell selective protein labeling is possible with azidonorleucine.\textsuperscript{81} In 2014, Smeekens and co-workers described a unique approach to analyze cell surface \textit{N}-glycoproteins comprehensively and site-specifically.\textsuperscript{82} For this purpose, they combined metabolic labeling, copper-free click chemistry, and mass spectrometry (MS)-based proteomics methods together and cells were fed a sugar analog containing an azido group, \textit{N}-azidoacetylgalactosamine. Azido functionalized glycoproteins on the cell surface were then clicked to dibenzocyclooctyne-sulfo-biotin via copper-free click chemistry under physiological conditions. Next, protein extraction followed by digestion was performed, followed by click chemistry to append biotin to labeled products, and finally glycopeptides with the biotin tag were enriched by NeutrAvidin conjugated beads. Deglycosylation of enriched glycopeptides was carried out by peptide-\textit{N}-glycosidase F in heavy water and eventually these products were tagged with \textsuperscript{18}O for MS analysis.
Similarly, functionalized monosaccharides can be introduced into cell surface glycans by means of promiscuous enzymes in the biosynthetic pathways of these biopolymers. Laughlin and co-workers reported labeled glycans by metabolic glyco-engineering and bioorthogonal copper-free click chemistry. For this purpose, first the cells or organisms were treated with azido tagged sugar, tetraacetylated $N$-azidoacetyl-D-mannosamine ($\text{Ac}_4\text{ManNAz}$) and incorporated by the glycan biosynthetic machinery to generate various glycoconjugates, which are ultimately located on the surface of the cells. Regardless of the route exploited, each enzyme involved in the installation process must tolerate the unnatural motif. For this reason, typical biophysical probes, such as fluorescein, cannot be used as direct modifications to metabolic substrates (that is, amino acids, lipids or sugars) as their relatively large size would interfere with enzymatic transformations. A small functional group is more likely to be tolerated by metabolic enzymes. Thus, to date, bioorthogonal chemical reporters have been non-native combinations of endogenous functionality (as discussed earlier) or small, abiotic functional groups that can slip through existing biosynthetic pathways. Therefore, metabolic labeling can be an advantageous tool for exploring production and trafficking of different biomolecules in cell systems that eventually will illuminate light on crucial cellular processes.
CHAPTER TWO
A CLICKABLE AND PHOTOCLEAVABLE LIPID ANALOG FOR CELL MEMBRANE DELIVERY AND RELEASE

The scientific data used in this chapter has been published by the authors: Shahrina Alam, Daiane S. Alves, Stuart A. Whitehead, Andrew M. Bayer, Christopher D. McNitt, Vladimir V. Popik, Francisco N. Barrera, and Michael D. Best

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2.1 Introduction

Lipids are characterized as attractive candidates for the delivery of drugs and imaging agents since they are biocompatible and they are capable of targeting molecules to cells through actions including insertion and fusion involving cellular membranes. Therefore, advanced methods for covalent conjugation of active molecules onto lipids and liposomal surfaces have become popular in recent years. In this regard, bioconjugation approaches that allow for the selective attachment of molecules within the intricate environments of biological samples are particularly beneficial. This concept has been applied to covalently attach drug moieties onto lipid assemblies such as liposomes, enabling their anchoring onto the membrane surface and subsequent delivery to cells. Additionally, it is convenient to apply a controllable technique when molecules are covalently conjugated to the lipids to trigger the release of the active molecule from the membrane surface.
Recently, biorthogonal methods, such as click chemistry reactions, have been explored for the derivatization of lipids and membranes to attach molecules and exploit the selectivity and fast kinetics of these reactions under ambient conditions.\textsuperscript{11-13, 88-90} Initial methodologies have focused on developing the copper-catalyzed azide–alkyne cycloaddition (CUAAC) for membrane derivatization.\textsuperscript{40, 43, 91-96} However, this reaction has some serious concerns as the prerequisite copper catalyst can cause the decomposition of lipid membranes.\textsuperscript{97} For this reason, copper-free click chemistry has emerged as a powerful tool for lipid and membrane derivatization\textsuperscript{45, 98, 99}. Applying this concept, our group reported functionalization of membrane surfaces using copper-free click chemistry employing an azido-lipid conjugate to derivatize liposomal membranes.\textsuperscript{45} In addition, researchers have successfully attached thrombomodulin proteins to liposomes to enhance activity,\textsuperscript{98} and achieved the targeting of liposomes bearing trans-cyclooctene moieties to cancer cells via the pH low insertion peptide (pHLIP) via bioorthogonal reactions.\textsuperscript{99} The strain promoted azide-alkyne cycloaddition (SPAAC) is more suitable than other bioorthogonal protocols that are available. It is more advantageous than the Staudinger ligation method since neither alkyne nor azide group is readily oxidized unlike phosphines. Moreover, dibenzocyclooctyne reagents\textsuperscript{100} have been developed as robust reactants with desirable kinetic properties, and in particular azodibenzocyclooctyne reagents, termed ADIBO or DIBACO, benefit from fast kinetics and ease of synthetic preparation.\textsuperscript{30} This ring strained
cyclooctyne species promotes the copper free [3+2] dipolar cycloaddition without any toxic copper catalyst and exhibits comparable kinetics to the Cu-catalyzed reaction.

2.2 2-Nitrobenzyl Photoactivating Group in Light Triggering Release Techniques

Photo-induced release systems commonly revolve around a self-assembled system that undergoes a change in structure upon photocleavage or cleavage of a linker by which a drug is covalently attached to the nanocarrier.\textsuperscript{63-65} Light-initiated release is favorable as it is a general approach that can be controlled in a spatially resolved manner to stimulate release at desired target sites, and the 2-nitrobenzyl groups is among the best studied for photoinitiated bond cleavage.\textsuperscript{101} Prior work involving the incorporation of photocleavable groups into lipids has focused on light-initiated release of molecules from liposomes.\textsuperscript{102-108} The strategy adopted here is straightforward; molecules such as drugs are non-covalently encapsulated in the liposome and release is instigated by decomposition or disruption of the membrane. Nevertheless, non-covalent encapsulation of drug moieties often leads to significant background leakage, and therefore it can be desirable to covalently attach target molecules to lipids prior to triggering the release through bond cleavage.

Recently, our group employed the o-nitrobenzyl moiety to synthesize the biologically-inert and photocleavable PC analogue NB-PC, where the photosensitive 2-nitrobenzyl group was embedded within the acyl chain at the sn-
2 position to produce a photocleavable lipid with a structure similar to that of natural PC. This lipid was constructed in such a way that photocleavage would cause disruption of the sn-2 acyl chain, ultimately culminating in the formation of an LPC product that is a non-bilayer lipid, thereby overall perturbing membrane properties. Photolysis studies detected the release of the hydrophobic dye nile red solubilized by the liposome by monitoring fluorescence with a series of liposomal formulations. We studied different liposomes formulations with different percentages of NB-PC, PC, PE, cholesterol, and PEG-PE and established minimal background release in controls, which indicated that release efficacies correlated with the amount of NB-PC incorporated. Moreover, release was only minimally impacted by the inclusion of the lipids PE and cholesterol that possess disparate membrane properties. As a result, nile red could be released from liposomes composed of a wide variety of lipids through incorporation of NB-PC.

2.3 Design of Photocleavable and Clickable Lipid

In this project, we set out to design a lipid delivery system that contains both a copper-free clickable group for the attachment of molecules onto the liposome surface as well as a photocleavable group for release after targeting. The resulting compound is 2.1, containing an α-nitrobenzyl group that links the glycerolipid membrane anchor to the ADIBO unit by which cargo is attached. A schematic for cellular delivery and release using this compound is depicted in Scheme 2.1, which was initially assessed through the delivery of fluorescent
dyes using compound 2.1. Compound 2.1, either in individual or liposome form, can be incubated with cells, leading to cell entry through processes such as endocytosis or membrane fusion. Afterward, bioorthogonal copper-free click reaction can be performed to target azide-tagged molecules to cells by exploiting the ADIBO unit of 2.1. As an alternative, targeting groups could be clicked onto 2.1 prior to cell delivery or conjugation could be performed using liposomes containing 2.1 for subsequent delivery and fusion with cell membranes. In the example depicted in Scheme 2.1, derivatization with Azide-Fluor 488 (2.14) leading to triazole-linked product 2.15 was exploited for fluorescence-based analysis. Next, photocleavage through irradiation was accomplished with 350 nm light, which results in release of the clicked molecule either on the inside of cells or in the proximity of cellular membranes. This follows through the traditional cleavage of the benzylic position to produce aldehyde-lipid byproduct 2.16 as well as the released triazole-linked conjugated molecules, in this case 2.17.

2.4 Synthesis of Lipid Analog

The synthetic route to clickable and photocleavable lipid 2.1 entailed coupling of alkyne-bearing 2-nitrobenzene 2.2 to azido-lipid scaffold 2.3 via click chemistry, followed by reaction with ADIBO-carboxylic acid 2.4 (Scheme 2.2). For the synthesis of alkynyl 2-nitrobenzene 2.2, we followed previously reported literature by first protecting the amino group of 4-aminomethylbenzoic acid (2.5) as tert-butyloxycarbonyl (Boc) group to 2.6.
Scheme 2.1: Delivery of lipid analog 2.1 to cell membranes, followed by click reaction to attached functionality and later photocleavage to release the targeted cargo.

Next, a nitration reaction was carried out to synthesize 2.7. The Boc protecting group was then exchanged to the trifluoroacetate moiety of 2.8. These initial three steps benefitted from previously reported procedures. A carboxylic acid 2.8 was then coupled to propargylamine to introduce the clickable alkyne handle of 2.2. While we have previously reported azide-tagged lipid 2.3, a modified synthesis was developed to enhance yields by avoiding volatile intermediates. For the current route, S-glycerol acetonide (2.9) was converted to tosylate 2.10, as previously reported, followed by acetonide deprotection to diol 2.11, azide displacement to 2.12, and acylation with two stearic acid moieties to produce 2.3. Next CUAAC was employed to couple compounds 2.2 and 2.3 to form 2.13,
which is beneficial due to synthetic facility. Furthermore, prior evidence has suggested that the polarity of the triazole headgroup may make this moiety an effective polar lipid headgroup mimic that lacks the charge that makes phospholipids difficult to purify and characterize.\textsuperscript{109, 110} Finally, the Boc protecting group of 2.13 was deprotected using trifluoroacetic acid (TFA) and the resulting amine was coupled to ADIBO-carboxylic acid 2.13, synthesized as previously described,\textsuperscript{30} to access 2.1. Compound 2.4 was generously provided by Dr Vladimir Popik’s group at the University of Georgia, and was synthesized by Christopher Mcnitt in that group.

Scheme 2.2: Synthesis of a lipid analog 2.1 with a cyclooctyne and 2-nitrobenzyl group.
2.5 Solution Phase Fluorescence Detection of Liposome Derivatization

Results

We first studied lipid analog 2.1 by examining its efficacy for functionalizing liposomes through copper-free click chemistry. Liposome modification was tested through the reaction of liposomes with 3-azido coumarin, which was synthesized by Stuart Whitehead in the Best group. This is a fluorogenic dye that is not initially fluorescent, but for which emission properties are activated upon formation of the triazole ring via the click reaction. Here, unilamellar liposomes (extruded to ~200 nm) were prepared composed of 96 % PC and doped with 4% lipid 2.1. Standard procedures including hydration and extrusion as well as dynamic light scattering were used to formulate and characterize liposomes. Next, derivatization of liposomes with 2.18 was assessed in solution using a fluorimeter to track enhanced fluorescence resulting from click chemistry. The fluorescence of the liposomal solution increased after addition of 2.18 and finally plateaued after ~ 40 minutes, indicating the azido group of 2.18 successfully clicked onto cyclooctyne group of lipid analog 2.1 (Figure 2.1). Liposomes lacking 2.1 were additionally incubated with 2.18 as a control and fluorescence was detected over the same time period as seen in Figure 2.1. In this case, only a minor increase in fluorescence was observed, likely attributable to a slight enhancement of the fluorescence properties of 2.18 caused by some insertion into the hydrophobic membrane environment. This change in fluorescence of
2.18 in the presence of membranes explains the double curve character of the results seen from the study sample.

Figure 2.1: Change in fluorescence of azido-coumarin 2.18 in the presence of liposomes containing and lacking cyclooctyne lipid 2.1.

### 2.6 Microplate Fluorescence Liposome Derivatization Studies

Although the solution liposome assay is a convenient method for detecting fluorescence as a result of liposomes conjugation as a function of time, this study suffers from the background fluorescence due to 2.18 in the presence of membranes lacking 2.1. Thus, we introduced a surface immobilization assay as an alternative method for detecting liposome conjugation via 2.1 (Scheme 2.3). Our group has previously developed a fluorescence-based liposome
immobilization assay to monitor protein-membrane binding interactions. This surface-based analysis offers a beneficial strategy for characterizing receptor-ligand binding interactions that has been exploited for the study of DNA, protein and peptide, and to a lesser extent, lipid binding interactions.

Scheme 2.3: Principle of streptavidin coated microplate assay for immobilization of liposomes using reaction with 3-azidocoumarin to characterize membrane derivatization.

Moreover, for this immobilization assay, it has been demonstrated that liposomes are successfully anchored onto the surface of the microplate, and immobilization is driven by an incorporated biotin-lipid conjugate rather than through non-specific binding. In addition, a dye leakage assay was used to
show that liposomes remain intact on the surface. The strategy involves the incubation of the biotinylated liposome, which is immobilized onto the streptavidin-coated microplate. Another significant aspect of this assay is that the biotin anchor needs to be presented at some distance from the membrane surface, as liposomes are known to degrade upon contact with and fuse to certain surfaces.  

![Figure 2.2: Change in fluorescence of azido-coumarin 2.14 in the presence of microplate-immobilized liposomes following wash off of unreacted dye.](image)

To apply this assay to assess derivatization of lipid 2.1, liposomes containing this lipid were immobilized onto streptavidin-coated 96-well microplates through incorporation of biotin-functionalized phosphatidylethanolamine analog 2.19, previously synthesized and reported by our group.109 Following incubation of
liposomes with 2.18, the immobilization step enabled the removal of unreacted dye from the surface-bound liposomes after washing the wells with buffer solution. Liposome derivatization was assessed using a fluorescence microplate reader (excitation filter 360 ±40 nm, emission filter 440 ± 40 nm) to detect the enhanced fluorescence of 2.18 upon triazole formation with 2.1 incorporated into liposomes. As is evident from Figure 2.2, this experiment clearly displayed a dose-dependent response as an increase in liposome concentration leads to higher fluorescence values due to formation of fluorescent click conjugate upon reaction of 3-azido coumarin. This microplate based study confirmed successful liposome conjugation via copper-free click chemistry with lipid 2.1. In addition, the removal of unreacted dye yields a more traditional curve for product formation compared to the results obtained from the prior solution studies of liposome modification.

2.7 Fluorescence Imaging and Flow Cytometry Analysis of Fluorescence Labeling and Photocleavage using Saccharomyces cerevisiae Cells

With successful results from liposome studies, we next set out to assess delivery and release using live Saccharomyces cerevisiae cells (Scheme 2.1). This study is aimed at the examination of the lipid analog 2.1 for delivery and release of species in live cells using fluorescence microscopy. Fluorescence microscopy, a versatile technique, is widely used to examine a number of essential phenomena such as cellular delivery. In these experiments, cells were first cultured, incubated with 2.1, the fluorophore labeled species of interest was
added and, and the cells were localized on glass slides and cover glass by a fixation agent. By means of a xenon or mercury lamp, the system was irradiated with UV light. The emitted light was then imaged through a microscope objective.

Recently, Neef et al.\textsuperscript{115} reported the fluorogenic labeling of alkyne-containing phosphatidic acid (PA) probes. Delivery of these lipid probes to cell membranes was confirmed by fluorescence microscopic images. Similarly, our study assesses the delivery of the fluorescent lipid analog to membranes and release of cargo. Therefore, we next set out to assess delivery and release using live \textit{Saccharomyces cerevisiae} cells (Scheme 2.1). To do so, yeast cells were grown in the presence and absence of 2.1 in the glucose medium. After growing cells overnight up to OD\textsubscript{600} values (optical density of cells at 600 nm) of 1.2, cells were incubated with commercially available Azide-Fluor 488 (2.14), followed by a number of washes and finally cells were fixed onto slides. Next, these cell samples were analyzed using a fluorescence microscope to assess delivery of the fluorophore to cells, for which representative images are shown in Figure 2.3. Here, cells grown in the presence of lipid 2.1 (Figure 2.3A) result in significant fluorescence compared to control studies lacking 2.1 (Figure 2.3B), both of which were incubated with Azide-Fluor 488 and washed with 25\% DMSO/water to remove unreacted dye. These figures showcase the successful delivery of lipid 2.1 to cells and subsequent derivatization.
Figure: 2.3 Fluorescence images of yeast cells after click reaction and photocleavage.

A. cells grown in the presence of 2.1, B. cells grown in the absence of 2.1, and C. cells grown in the presence of 2.1 and irradiated for 12 hours, each of which was washed to remove unbound dye prior to imaging. D-F. Transmission Images showing cell population for the three experiments, respectively.
To assess release via photocleavage, cells were photolyzed using a Rayonet photoirradiator for different periods of time. Next, cells were washed once with 5% DMSO in water and twice with 20% DMSO in water to remove unreacted dye (not attached to lipid 2.1 embedded within cell membranes). After fixing the cells with ice-cold methanol, they were subjected to confocal fluorescence microscopy. A representative image in Figure 2.3C taken after 12 hours of photocleavage demonstrates that fluorescence is significantly decreased following photocleavage. Control studies in which the labeling dye was subjected to irradiation conditions used for photolysis indicated that photobleaching was not occurring as no decrease in fluorescence was observed (data not shown). In addition, transmission images (Figures 2.3D-F) are included for the three described images, respectively, showing the presence of cells in each sample.

For quantitative evaluation of delivery and release in vivo studies, we employed flow cytometry-based analysis, with results shown in Figure 2.4. Figure 2.4A illustrates a bar graph of fluorescence for control cells that were not treated with 2.1 or 2.14 (unstained), study cells that were incubated with 2.1, clicked with 2.14 and washed (stained), and stained cells after photocleavage for 1, 2, 3, and 12 hours followed by washing. From this graph it is evident that clicked cell samples exhibited maximum fluorescent cells, whereas photocleavage yielded a decrease to 70%, 60%, 57%, and 42% of the original fluorescence of the unstained sample after 1, 2, 3, and 12 hours, respectively.
Figure 2.4: Results from flow cytometry of labeled cells for quantitative analysis. A. Bar graph depicting fluorescence detected for cells not treated with 2.1 (unstained), cells treated with 2.1 (stained), and treated cells irradiated for 1, 2, 3, and 12 hours, each of which was washed prior to fluorescence imaging. B. Flow cytometry results for cells that were unstained (red), after click with 2.14 (blue), and after photocleavage for 3 (green) and 12 (orange) hours.
In addition, representative plots of fluorescence signal observed from flow cytometry analysis of the different described samples are shown in Figure 2.4B.

2.8 MTS Assay Results and Mass Spectrometry Evidence for Delivered Camptothecin to T24 Cells

Finally, our last study was aimed at evaluating compound 2.1 for delivery and release of an anti-cancer therapeutic with human cancer cells towards a real world application of this compound as a drug delivery vehicle. Here, we were interested in determining whether compound 2.1 would allow clicked cargo to be released inside the cell and thus exhibit medicinal properties. Therefore, we decided to employ camptothecin (CPT, 2.22), a potent anti-cancer drug that is challenging to deliver due to its poor solubility. To deliver this compound via copper-free click chemistry, we designed clickable analog 2.20 containing an azido-hexanoyl chain esterified to the free hydroxyl group of the drug (Scheme 2.4). Moreover, this analog enables triggered release without requiring photocleavage since the ester linker can be hydrolyzed following cell entry by intracellular esterases, thereby releasing camptothecin itself. For this purpose, HeLa cells were incubated with PC liposomes doped with 6% of lipid 2.1 in the presence and absence of camptothecin-azide 2.20 (CPT-N$_3$). This experiment was performed in collaboration with Daiane S. Alves in Francisco Barrera’s lab.

Here, liposomes containing 2.1 were incubated with CPT-N$_3$ 2.20 for attachment onto the liposome surface, treated with cells, and cell viability was then assessed through the MTS assay. This produced liposome-immobilized conjugate 2.21,
which was confirmed by mass spectrometry. Camptothecin itself was studied using a DMSO vehicle as a positive control, and all cell viability studies were performed using a standard MTS assay. In these studies, liposomes containing 2.1 alone did not influence cell viability, but those that were pre-incubated with added 2.20 ranging from 10-50 µM led to a dose-dependent decrease in living cells (Figure 2.5). Compound 2.20 alone was not viable for studies since it is not soluble in aqueous solution. These results demonstrate the delivery of camptothecin to cancer cells using compound 2.1.

Scheme 2.4: Liposomal delivery of camptothecin to Hela cells.

These studies also explain the phenomena by which lipid 2.1 interacts with membranes and where release occurs. Two possible mechanisms for release via photocleavage could be responsible for delivery to cells. First of all, lipid 2.1 conjugated to cargo could use its hydrophobic tail to anchor onto the outer leaflet of cell membrane, which would lead to a high local concentration of the drug around the periphery of cells.
Figure 2.5: MTS assay in human cancer cell line indicates that CPT-N₃ reduces cell proliferation in a dose-dependent manner.

HeLa cells were treated with vehicle (Ct (-)-DMSO 0.01%), CPT (22, 50 µM), Liposomes control (Lip) and liposomes pre-incubated with CPT-N₃ 22 (Lip+CPT-N₃) at different CPT-N₃ concentrations (10 µM, 25 µM and 50 µM) for 48 hrs. Cell proliferation was measured by MTS assay and the graph represents the percentage relative to the control CT(-). The results represent the mean of 3 independent experiments. Statics t-test: * p<0.05 and ** p<0.001 two populations relative to CT (-). The error bars shown correspond to the standard deviation.
Alternatively, after being incorporated into the outer leaflet of cell membranes, lipid 2.1 bearing cargo could flip across the membrane into the inner leaflet prior to release. The second mechanism would be beneficial since lipid 2.1 could facilitate the membrane translocation of the cargo, although this release process is highly dependent on the properties of the cargo that is being delivered. Finally, the liposomes could enter cells by endocytosis. The camptothecin esterase release results provide evidence to support that this molecule conjugated to lipid 2.1 do enter cells since this would be required in order for the release by esterase hydrolysis to occur. Delivery of CPT-N\textsubscript{3} and hydrolysis by esterases has been further confirmed after lipid extraction of the cells; cells incubated with 2.21 were lysed, extracted with hydrochloric acid (2N) and the aqueous non-lipidic component was analyzed by mass spectrometry, which clearly showed a peak for a derivative of camptothecin due to acidic hydrolysis at m/z value 341.80. This peaks also observed when natural camptothecin (2.22) was subjected to the same acidic hydrolysis.

2.9 Conclusion

After synthesizing lipid analog 2.1 containing a click reporter group and a photocleavable moiety, we successfully demonstrated that it can be conjugated when incorporated into liposomes via two different fluorescence assays involving fluorogenic dye conjugation and a liposome immobilization assay. Fluorescence microscopy studies corroborated by flow cytometry showed that this compound could be exploited to deliver fluorescent cargo to cells, and subsequent
irradiation with UV light triggered release. In drug delivery studies, the MTS assay confirmed the delivery of camptothecin into Hela cells using lipid analog 2.1, leading to a dose-dependent decrease in cell viability. While this compound was shown to be successful for delivery and release in vitro, the 2-nitrobenzyl group in 2.1 absorbs light in the ultraviolet (UV) wavelength range that hinders its applicability since many biomolecules absorb irradiation in this range, leading to their photodecomposition as well as the inability of the beam to penetrate tissue. Therefore, in the future we will design and develop two photon initiated systems that are activated in the near-IR (NIR) range, which benefit from enhanced penetration and decreased damage to cells.

2.10 Experimental

Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. PC (L-α-Phosphatidylcholine from chicken egg) was purchased from Avanti Polar Lipids, Inc. and 4(aminomethyl)benzoic acid was purchased from Chem Impex International. Dry solvents were obtained from a Pure solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian Mercury 500 and 600 spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF and ABI Voyager DE Pro MALDI mass spectrometers with high-resolution capabilities. Optical rotation values were obtained using a Perkin-Elmer 241 polarimeter. Liposome extruder and
polycarbonate membranes were obtained from Avestin (Ottawa, Canada). Ultrapure water was purified via a Millipore water system (≥ 18 MΩ·cm triple water purification system). 4-(((Tert-butoxycarbonyl)amino)methyl)-3-nitrobenzoic acid (2.8)\textsuperscript{104} and 6-azidohexanoic acid (2.23)\textsuperscript{117} were synthesized from 4-(aminomethyl)benzoic acid (2.5) and 6-bromohexanoic acid, respectively, according to prior literature procedures. Biotin-PE 2.19 was synthesized as previously described.\textsuperscript{109} Solution fluorescence studies were performed using a Perkin Elmer LS55 fluorimeter. For photocleavage, samples were irradiated with a Rayonet Preparative Type RS photoreactor while suspended in a cuvette covered with a Pyrex beaker as a filter. Disposable polystyrene cuvettes were purchased from Fisher scientific. Microplate-based fluorescence measurements were performed using a BioTek synergy 2 multi-detection microplate reader. Black reacti-bind streptavidin high binding capacity (HBC) coated 96-well microplates were purchased from Pierce Biotechnology (Rockford, IL). Cell images were captured using a Leica SP2 laser scanning confocal microscope. Fluorescent cell counting was performed using a BD FACS (Fluorescence Activated Cell Sorting) Calibur flow cytometer.
**tert-Butyl-2-nitro-4-(prop-2-yn-1-ylcarbamoyl)benzylcarbamate (2.2).** To 4-(((Tert-butoxycarbonyl)amino)methyl)-3-nitrobenzoic acid (2.8) (0.718 g, 2.40 mmol) dissolved in 200 mL of dichloromethane in a 500 mL round-bottomed flask was added dicyclohexylcarbodiimide (DCC) (0.743, 3.60 mmol), and N,N-dimethylaminopyridine (DMAP) (0.147 g, 1.20 mmol). After 30 min, propargylamine (185 µL, 2.88 mmol) was added. The reaction was then allowed to stir overnight, after which it was washed with water, and the aqueous portion was extracted with dichloromethane (3 x 25 mL). The organic layers were then combined and dried with magnesium sulfate, filtered and concentrated by rotary evaporation. Column chromatography using gradient elution with 30-50% ethyl acetate-hexanes gave 2.2 as a yellow solid (638 mg, 79% yield). $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 8.45 (s, 1H), 8.06 (d, $J = 3.3$ Hz, 1H), 7.63 (d, $J = 3.6$ Hz, 1H), 4.55 (s, 2H), 4.13 (d, $J = 2.5$ Hz, 2H), 2.58 (t, $J = 2.5$ Hz, 1H), 1.41 (s, 9H). $^{13}$C NMR (126 MHz, CD$_3$OD): $\delta$ 165.46, 156.59, 147.87, 138.06, 134.03, 132.15, 130.23, 124.01, 79.98, 79.05, 77.29, 71.18, 48.54, 41.80, 33.57, 29.29, 28.02, 25.48, 24.79. HRMS-DART [M-H]$^-$ calcd for C$_{16}$H$_{19}$N$_3$O$_5$, 332.1246; found 332.1243.

![Diagram of the reaction](image-url)
(R)-2,3-dihydroxypropyl-4-methylbenzenesulfonate (2.11). To (S)-(+) 1,2-Isopropylideneglycerol (2.10) 1.00 g, 3.49 mmol) dissolved in 50 mL of methanol in a 500 mL round-bottomed flask was added 125 mL of 0.50 N hydrochloric acid, and the reaction mixture was allowed to stir for 12h. The excess HCl was neutralized by adding solid sodium bicarbonate until carbon dioxide gas release was complete. Next, the crude mixture was passed through celite, filtered off, and the methanol was removed by rotary evaporation. Compound 2.11 was then eluted with 100% ethyl acetate using normal phase silica gel column chromatography and obtained as colorless oil (816 mg, 95% yield). $^1$H NMR (500 MHz, CDCl$_3$): δ 7.77 (d, $J$ = 7.8 Hz, 2H), 7.33 (d, $J$ = 7.8 Hz, 2H), 4.03 (s, 2H), 3.92 (s, 1H), 3.57 (m, 2H), 2.42 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$): δ 145.22, 132.21, 130.02, 127.95, 70.94, 69.68, 62.77, 21.62. HRMS-DART [M+H]$^+$ calcd for C$_{10}$H$_{14}$O$_5$S, 247.0640, found 247.0644.
(S)-3-(4-(((tert-butoxycarbonyl)amino)methyl)-3-nitrobenzamido)methyl)-1H-1,2,3-triazol-1-yl)propane-1,2-diyl distearate (2.13). To alkyne 2.2 (0.400 g, 1.20 mmol) dissolved in 125 mL tetrahydrofuran-water (1:1) in a 250 mL round-bottomed flask was added azide 2.3 (0.780 g, 1.20 mmol). Next, copper sulfate pentahydrate (0.390 g, 1.56 mmol) and lastly sodium ascorbate (0.475 g, 2.40 mmol) were added. After overnight stirring, the solution was then concentrated and dried under high vacuum. Column chromatography with 5% methanol/dichloromethane gave product 2.13 as a light yellow solid (575 mg, 50% yield). $^1$H NMR (500 MHz, 85% CDCl$_3$-CD$_3$OD): $\delta$ 9.06 (s, 1H), 8.54 (s, 1H), 8.13 (d, $J = 8.5$ Hz, 1H), 7.85 (s, 1H), 7.70 (d, $J = 5.2$ Hz, 1H), 6.54 (s, 1H), 5.49 – 5.37 (m, 1H), 4.71 – 4.58 (m, 4H), 4.38 (dd, $J = 12.0$, 3.8 Hz, 2H), 4.10 (dd, $J = 7.1$, 4.8 Hz, 2H), 2.41 – 2.24 (m, 4H), 1.69 – 1.53 (m, 4H), 1.45 (s, 9H), 1.27 (s, 56H), 0.89 (t, $J = 6.5$ Hz, 6H). $^{13}$C NMR (126 MHz, 85% CDCl$_3$-CD$_3$OD): $\delta$ 173.85, 173.20, 165.83, 156.71, 154.58, 148.24, 145.05, 138.24, 134.53, 132.39, 131.00, 124.46, 77.82, 77.56, 69.68, 62.41, 50.48, 49.00, 35.15, 34.23, 33.94, 32.62, 32.16, 31.07, 29.92, 29.72, 29.59, 29.35, 29.25, 28.43, 26.25, 25.84, 25.07, 24.96, 22.90, 14.19. HRMS-DART [M+H]$^+$ calcd for C$_{55}$H$_{94}$N$_6$O$_9$, 983.72, found 983.73. $[\alpha]^{24.0}_{D}$ -3.33$^\circ$ (c 1, CHCl$_3$).
**ADIBO−Nitrophenyl−Lipid (2.1).** In a 50 mL round-bottom flask, compound **2.13** (42.6 mg, 0.113 mmol) was dissolved in 10 mL dichloromethane/trifluoroacetic acid (1:1) and stirred for 5 h. Excess acid was removed by rotary evaporation with dichloromethane. The free amine was re-dissolved in 15 mL dichloromethane. To this solution were added hydroxybenzotriazole (HOBT, 15.3 mg, 0.113 mmol) and O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU, 42.8 mg, 0.113 mmol). After 30 min, a mixture containing ADIBO **2.4** (0.038 g, 0.113 mmol) dissolved in 10 mL dichloromethane with diisopropylethylamine (92.6 µL, 0.566 mmol) was added to the reaction mixture, which was then allowed to stir at rt under nitrogen atmosphere overnight. The next day, the crude mixture was passed through celite and then washed with 50 mL water. The collected aqueous phase was next washed with dichloromethane (3 x 20 mL). The organic layers were then combined and dried with magnesium sulfate. After filtration and concentration, column
chromatography was carried out through gradient elution with 7-10% methanol-dichloromethane, which provided lipid 2.1 as a yellow-white oily solid (53.44 mg, 38% yield). $^1$H NMR (500 MHz, 85% CDCl$_3$-CD$_3$OD): $\delta$ 8.79 (t, $J = 5.7$ Hz, 1H), 8.54 (d, $J = 1.9$ Hz, 1H), 8.05 (d, $J = 12.7$ Hz, 1H), 7.79 (s, 1H), 7.67 (d, $J = 7.6$ Hz, 1H), 7.61 (d, $J = 8.1$ Hz, 1H), 7.42 – 7.37 (m, 3H), 7.34 – 7.26 (m, 4H), 6.89 – 6.85 (m, 1H), 5.43 – 5.37 (m, 1H), 5.16 – 5.11 (m, 1H), 4.73 – 4.68 (m, 2H), 4.65 – 4.57 (m, 4H), 4.33 (dd, $J = 12.2$, 4.2 Hz, 1H), 4.08 (dd, $J = 12.2$, 5.4 Hz, 1H), 3.37 (dq, $J = 4.2$, 1.4, 0.9 Hz, 1H), 3.26 – 3.21 (m, 1H), 3.15 (ddd, $J = 8.6$, 7.5, 0.9 Hz, 2H), 2.36 – 2.28 (m, 5H), 2.05 – 1.98 (m, 1H), 1.58 (dq, $J = 32.6$, 6.9 Hz, 4H), 1.41 (dd, $J = 10.1$, 3.7 Hz, 4H), 1.26 (s, 56H), 0.88 (t, $J = 6.8$ Hz, 6H). $^{13}$C NMR (151 MHz, 85% CDCl$_3$.CD$_3$OD): $\delta$ 173.64, 173.28, 172.97, 172.60, 172.25, 171.95, 165.55, 151.05, 148.17, 147.95, 144.92, 137.24, 134.35, 132.25, 132.13, 130.82, 129.15, 128.84, 128.67, 128.40, 128.06, 127.33, 125.74, 124.33, 124.25, 123.03, 122.59, 114.87, 107.80, 77.35, 69.51, 62.24, 60.77, 55.74, 54.87, 50.35, 49.12, 35.55, 35.08, 34.52, 34.12, 32.05, 31.71, 31.25, 29.83, 29.62, 24.91, 22.80, 14.16. MALDI-MS: [M+Na]$^+$ calcd for C$_{72}$H$_{104}$N$_8$O$_{10}$, 1263.78; found 1263.77. $[^{\alpha}]_D^{24.0} +1.2^\circ$ (c 1, CHCl$_3$).
(S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyran[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 6-azidohexanoate) (2.20). To 6-azidohexanoic acid (2.23) (1.13 g, 7.20 mmol) dissolved in 30 mL dry dichloromethane in a 500 mL round-bottomed flask was added dimethylaminopyridine (DMAP) (0.88 g, 7.20 mmol), N,N′-diisopropylcarbodiimide (DCC) (1.13 mL, 7.20 mmol) and Scandium(III) triflate (0.71 g, 1.44 mol). The reaction mixture was cooled to -8 ºC. Lastly, (S)-(−)-Camptothecin (2.22) (0.83 g, 2.38 mmol) was added and the resulting mixture was allowed to stir for 18 h. The reaction mixture was washed with 0.1 N HCl and 1% (v/v) sodium bicarbonate solution. Residual water was dried over magnesium sulfate and then was passed through celite and filtered off. Compound 2.20 was then eluted with 100% ethyl acetate using normal phase silica gel column chromatography and obtained as a white solid (712 mg, 61% yield). $^1$H NMR (500 MHz, CDCl$_3$): δ 8.40 (s, 1H), 8.22
(dd, $J = 8.5$, 0.8 Hz, 1H), 7.96 – 7.93 (m, 1H), 7.86 – 7.83 (m, 1H), 7.68 (m, $J = 8.1$, 6.9, 1.2 Hz, 1H), 7.21 (s, 1H), 5.68 (d, $J = 17.2$ Hz, 1H), 5.41 (d, $J = 17.2$ Hz, 1H), 5.29 (dd, $J = 3.5$, 1.2 Hz, 2H), 3.22 (td, $J = 6.9$, 2.7 Hz, 2H), 2.52 (dt, $J = 10.4$, 7.3 Hz, 2H), 2.29 (dd, $J = 14.0$, 7.4 Hz, 1H), 2.18 – 2.13 (m, 1H), 1.72 – 1.67 (m, 2H), 1.60 (ddd, $J = 11.1$, 5.6, 2.8 Hz, 1H), 1.46 – 1.40 (m, 1H), 1.25 (s, 2H), 0.98 (t, $J = 7.5$ Hz, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 172.39, 167.52, 157.33, 152.34, 148.84, 146.21, 131.21, 129.52, 128.45, 128.22, 128.17, 128.04, 120.28, 95.89, 75.82, 67.10, 51.12, 49.91, 33.57, 31.84, 28.49, 26.03, 24.17. HRMS-DART [M+H]$^+$ calcd for C$_{26}$H$_{25}$N$_5$O$_5$, 488.1933, found 488.1935.

**Liposome preparation.** Stock solutions were made of synthetic lipid 2.1, PC and biotin lipid 2.19 for microplate studies. Examples of each stock solution used are as follows: 80.0 mg lipid 2.1, 17.2 mg PC, and 3.90 mg 2.19 were each weighed into separate vials and dissolved in 500 µL chloroform to form stock solutions of 1.29 mM lipid 2.1, 1.67 mM biotin-PE, and 44.7 mM PC. Using these stock solutions, 55.9 µL lipid 2.1 stock (2%), 5.40 µL biotin-PE stock (1%), and 78.2 µL PC stock (97%) were combined in a glass vial. For solution-phase fluorimeter studies, a representative sample included 31.0 µL of lipid 2.1 stock (4%) and 21.5 µL of PC stock (96%), while a control sample contained 22.4 µL of PC stock (100%). In each case, the chloroform solvent was removed under a stream of nitrogen and the resulting mixture was placed under vacuum overnight. The dried lipids were then hydrated by adding 500 µL of HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4), and rotated on a rotary evaporator for 60 min at 40 ºC.
The liposomes were then subjected to 10 freeze-thaw cycles and extruded (21 times) to obtain uniform size and lamellarity using an extruder containing a 200 nm polycarbonate filter.

**Solution phase fluorescence detection of liposome derivatization.** A 2.40 mM solution of azido-coumarin 2.18 in dimethylsulfoxide was prepared. 4.00 μL of this solution were then diluted with 1.88 mL of HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4), placed into a cuvette and sealed with parafilm to minimize atmospheric exposure. After an initial fluorescence scan (λ<sub>ex</sub> = 350 nm; λ<sub>em</sub> = 461 nm), 120 μL of control liposomal solution (2 mM, 100 % PC) was added and fluorescence readings were recorded for 45 min. The same procedure was followed for the study sample, except 120 μL of liposomal solution (2 mM, 96% PC and 4% lipid 2.1) were added, followed by fluorescence scanning over time.

**Microplate fluorescence liposome derivatization studies.** A 400 μM solution of azido-coumarin 2.18 in dimethylsulfoxide was prepared. In a 96-well streptavidin-coated microplate, 200 μL of wash buffer (0.5X PBS, pH 7.4, 0.56 mM phosphates) was added to each row to be used. The plates were then shaken for 30 min, after which the wash buffer was removed. Next, 0, 1, 2.5, 5.00, 7.50, 10.0, 15.0, 20.0, and 25.0 μL of liposome solution (2 mM, 97%PC, 2% lipid 2.1 and 1% 2.19) and 187.5, 198.5, 169.3, 192.3, 188.8, 177.5, 170, 162.5 μL HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) were added to the appropriate wells to produce final liposome concentrations of 0, 10, 25, 50, 75,
100, 150, 200 and 250 μM in separate wells. In addition, 12.5 (control), 0.50, 1.25, 2.50, 3.75, 5.00, 7.50, 10.0, 12.5 μL azido-coumarin **2.18** solution (5 equivalents, 400 μM solution) were added to each well to a total volume of 200 μL. These solutions were incubated, with shaking, for 3h at rt. Following incubation, the solutions were removed and each well was washed with 3 x 200 μL wash buffer and 200 μL HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) was then added. Lastly, fluorescence was measured using a microplate reader with a 360 nm (± 40 nm) excitation filter and 460 nm (± 40 nm) emission filters.

**Saccharomyces cerevisiae cells.** A YPD (yeast extract peptone dextrose) agar plate was inoculated with *S. cerevisiae* strain 5288 W CT and incubated at 30 °C for 24h. Next, 5 mL of YPD media were inoculated with a single colony obtained from the agar plate and incubated on a shaker at 300 rpm at 30 °C with good aeration for 15h. Next, 500 μL aliquots from this culture were transferred into two detergent-free 250 mL Erlenmeyer flask containing 25 mL of fresh YPD medium with (study) and without (control) lipid analog **2.1** (100 μM, 300 μL), both of which were incubated on a shaker at 300 rpm at 30 °C for 16 to 17h. For good aeration, the medium constituted of no more than one-fifth of the total flask volume. Thereafter, the cell concentration was adjusted spectrophotometrically to approximately 10⁶ CFU/mL. Finally, 1 mL of the cell solution was transferred into eppendorf tube, centrifuged at 9000 rpm for 4 min, and then stored at –80 °C.
Fluorescence Imaging and Flow Cytometry Analysis of Fluorescence Labeling and Photocleavage using Saccharomyces cerevisiae cells. Two 1 mL samples of S. cerevisiae cells, one treated with compound 2.1 (study) and one untreated (control), were incubated with 100 µM Azide-Fluor 488 dimethylsulfoxide (DMSO) solution overnight at rt. Next, the cell samples were centrifuged for 3 min at 2500 rpm, the dye solutions were removed, and the cells were washed once with 5% DMSO in water and twice with 20% DMSO in water, with vortexing and centrifugation at 2500 rpm for 3 min performed in between each washing step. Next, 1 mL ice-cold methanol was added to each tube, and after incubating for 10 min at 0 °C, the sample was centrifuged. Next, 1 mL of Phosphate Buffered Saline solution (PBS, pH 7.4, 1.19 mM phosphates) was used to wash the fixed cell samples, which were then centrifuged at 2500 rpm for 3 min. Cells were then subjected to fluorescence microscopy. To assess photocleavage, 200 µL of clicked cell sample were added into a disposable cuvette and 800 µL HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4), were added. For photocleavage, cells were irradiated with 350 nm light while suspended and covered by Pyrex beakers between four 350 nm bulbs in a Rayonet Preparative Type RS photoreactor. This was done for different time periods (1, 2, 3, 4 and 12h) to assess photocleavage over time. After photoirradiation, the cell samples in buffer were transferred into eppendorf tubes and centrifuged at 2500 rpm for 3 min, and then washed 7 times with 1 mL of 20% DMSO/water. Each time they were vortexed and centrifuged at 9000 rpm for 4
Lastly, the cells were washed with 1 mL of 1X PBS buffer (pH 7.4, 1.19 mM phosphate buffer), and fluorescence microscope images after photocleavage were then obtained. For flow cytometry analysis, cell samples were analyzed using a BD FACS (Fluorescence Activated Cell Sorting) Calibur flow cytometer (Becton, Dickinson and Company, New Jersey, US) equipped with two lasers (488 nm and 635 nm). Green fluorescence (FL-1) was measured for all cell samples. Populations of the yeast cells were identified and gated according to the fluorescent intensity. Data acquisition was performed and analyzed using FlowJo software. For each sample analyzed in the flow cytometer, at least 10,000 events were acquired.

**Cell proliferation assay (MTS).** HeLa cells were cultured in a humidified incubator under 5% CO₂ in DMEM medium (Invitrogen) supplemented with 10% of Fetal Bovine Serum (Invitrogen). The cell viability was measured using the CellTiter 96 ®Aqueous One Solution (Promega) according to the manufacturer’s protocol. Briefly, cells were seeded (10⁴ cells per well) 2 days prior to the experiments to a 96 well plate, and exposed to vehicle (DMSO and liposomes), CPT, CPT-N₃ or CPT-N₃ + Liposomes mix at different concentrations of CPT, CPT-N₃ (10 µM, 25 µM and 50 µM) and incubated for 48h. The liposomal solution consisted of 50 µM lipid analog 2.1 formed as 8.5 mM liposomes (94% PC, 6% lipid analog 2.1). The MTS assay was performed in 100 µL of DMEM phenol red free medium (Invitrogen) in each well and 20 µL of the CellTiter solution was added to the samples, after which the plate was placed in a 37 °C
incubator with 5% CO₂ until it reached the desired color. The absorbance at 490 nm was measured in a plate reader (Synergy 2, Biotek). The results are representative of 3 independent experiments, performed in quadruplicate. The inhibition of cell proliferation was expressed as the percentage of vehicle control (0.01% DMSO in the culture medium for CPT).

Linear alkyne lipid (2.25). In a 50 mL round-bottom flask, compound 2.13 (42.6 mg, 0.113 mmol) was dissolved in 10 mL dichloromethane/trifluoroacetic acid (1:1) and stirred for 5h. Excess acid was removed by rotary evaporation with dichloromethane. The free amine was re-dissolved in 15 mL dichloromethane. To this solution were added hydroxybenzotriazole (HOBt, 15.5 mg, 0.113 mmol) and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexa-fluorophosphate (HBTU, 43.2 mg, 0.113 mmol). After 30 min, a mixture containing 2.24 (0.015 g, 0.113 mmol) dissolved in 10 mL dichloromethane with diisopropylethylamine (95.6 µL, 0.571 mmol) was added to the reaction mixture, which was then allowed to stir at
rt under nitrogen atmosphere overnight. The next day, the crude mixture was passed through celite and then washed with 50 mL water. The collected aqueous phase was next washed with dichloromethane (3 x 20 mL). The organic layers were then combined and dried with magnesium sulfate. After filtration and concentration, column chromatography was carried out through gradient elution with 7-10% methanol-dichloromethane, which provided lipid **2.25** as a yellow-white oily solid (15.45 mg, 28% yield). $^1$H NMR (500 MHz, 85% CDCl$_3$-CD$_3$OD): δ 8.92 (t, J = 5.7 Hz, 1H), 8.60 (d, J = 1.94 Hz, 1H), 8.12 (d, J = 12.89 Hz, 1H), 7.80 (s, 1H), 7.61 (d, J = 7.6 Hz, 1H), 5.41-5.39 (m, 1H), 4.84 (s, 2H), 4.66 – 4.57 (m, 5H), 4.33 (dd, J = 12.2, 5.4 Hz, 1H), 4.08 (dd, J = 11.7, 4.3 Hz, 1H), 3.36 (dq, J = 4.2, 1.4, 0.9 Hz, 2H), 2.35 (t, J = 7.6 Hz, 2H), 2.30 (t, J = 7.6 Hz, 2H), 1.59 (m, 4H), 1.26 (s, 56H), 0.88 (t, J = 6.8 Hz, 6H). $^{13}$C NMR (151 MHz, 85% CDCl$_3$-CD$_3$OD): δ 174.05, 173.14, 165.58, 148.50, 144.94, 135.45, 135.06, 132.47, 130.78, 124.76, 69.75, 62.47, 50.52, 41.15, 34.27, 32.20, 29.96, 29.75, 29.62, 29.52, 29.39, 29.29, 25.12, 25.01, 22.93, 14.20.
2.11 List of Spectra

Spectra 2.1: $^1$H NMR and $^{13}$C NMR of compound 2.2. tert-Butyl-2-nitro-4-(prop-2-yn-1-ylcarbamoyl)benzylcarbamate.
Spectrum 2.2: Mass Spectrum of compound 2.2. tert-Butyl-2-nitro-4-(prop-2-yn-1-ylcarbamoyl)benzylcarbamate.
Spectra 2.3: $^1$H NMR and $^{13}$C NMR of compound 2.11. (R)-2,3-dihydroxypropyl-4-methylbenzenesulfonate.
Spectrum 2.4: Mass Spectrum of compound 2.11. (R)-2,3-dihydroxypropyl-4-methylbenzenesulfonate.
Spectra 2.5: $^1$H NMR and $^{13}$C NMR of compound 2.13. (S)-3-((4-(((tert-butoxycarbonyl)amino)methyl)-3-nitrobenzamido)methyl)-1H-1,2,3-triazol-1-yl)propane-1,2-diyl distearate.
Spectra 2.6: $^1$H NMR and $^{13}$C NMR of compound 2.1. ADIBO–Nitrophenyl–Lipid.
Spectra 2.8: $^1H$ NMR and $^{13}C$ NMR of compound 2.20. (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 6-azidohexanoate).
Spectrum 2.9: Mass Spectrum of compound 2.20. (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 6-azidohexanoate).
Spectrum 3.0: Mass spectrum of released fluorophore 2.17 following treatment of \textit{S. cerevisiae} cells with lipid 2.1, fluorophore 2.14 and photocleavage.
Spectrum 3.1: Mass spectrum of camptothecin-lipid conjugate formed following the incubation of lipid 2.1 with CPT-N$_3$ 2.20.
Spectrum 3.2: Mass spectrum of released camptothecin obtained following acidic extraction of Hela cells that were treated with camptothecin-lipid conjugate 2.21.
Spectrum 3.3: Mass spectrum obtained from a standard solution of camptothecin subjected to acidic extraction.
Spectra 3.4: $^1$H NMR and $^{13}$C NMR of compound 2.25. Linear alkyne lipid.
CHAPTER THREE
METABOLIC LABELING OF PHOSPHATIDYLSEERINE LIPIDS IN CELLS USING CLICKABLE SERINE ANALOGS FOR TARGETED LIPOSOME DELIVERY

This chapter shows data which is currently being prepared for submission to a journal paper by the following authors.

3.1 Importance of PS in Cell Membranes

In addition to acting as the primary components of cell membranes, lipids and proteins play important roles in cell–cell-signaling that are of extensive interest. Furthermore, lipids control many critical biological pathways, and thus aberrant lipid biosynthesis and activity commonly correlate with diseases including cancer.\textsuperscript{118-121} For example, phosphatidylserine (PS) is an important biomarker that participates in diverse biological processes. PS acts as a ligand for a number of proteins, thereby directing membrane association of these proteins through non-covalent protein–lipid binding interactions.\textsuperscript{122} One particularly well studied example is Annexin A5,\textsuperscript{123} a protein that participates in PS membrane translocation and is involved in key anti-coagulation events,\textsuperscript{124, 125} antiphospholipid antibody syndrome,\textsuperscript{126, 127} and has been used for cancer imaging applications.\textsuperscript{128}
While changes in lipid composition of cell membranes during carcinogenesis have been heavily explored, very little insight has been acquired. Nevertheless, in recent years lipidomics in cancer research has gained more attention. For example, the outer leaflets of eukaryotic plasma membranes are chiefly composed of neutral phospholipids, such as phosphatidylcholine (PC) and sphingomyelin (SM). On the other hand, negatively charged phospholipids including phosphatidylserine (PS) as well as phosphatidic acid (PA) are normally found in the inner leaflet. Interestingly, during apoptosis and the onset of certain cancers, PS is translocated to the outer leaflet of the cell membrane. This may be due to the reduced activity of phospholipid translocase enzymes may perturb this asymmetry. The function of ATP dependent phospholipid translocase is to transport PS and PE between bilayer leaflets and has been found to be vulnerable to oxidative injury. Another possible reason might be the activation of a scramblase triggered by increased intracellular Ca\textsuperscript{2+} levels, which leads to non-specific movement of phospholipids. Thus, loss of asymmetry causes distribution of the negatively charged PS on the external surface of cancerous and other pathological cells as well as apoptotic cells.

As a result of this phenomenon, PS represents a powerful target for drug delivery and biomarker for identifying cancer cells. In spite of the crucial role of lipids such as PS in metabolic activities, it is still a major challenge to follow the biosynthesis of these metabolites at their sites of origin, essentially due to the complexity of lipid biosynthetic pathways, in which numerous molecules are
continually undergoing interconversion within the complex environment of cellular membranes. In this project, we describe the development of clickable serine analogs that can be used to produce tagged versions of PS in cells. This is envisioned as a powerful tool for tracking the biosynthesis and trafficking of PS in cells. Furthermore, due to the presentation of PS on the outer surfaces of cancer cells, an additional goal is to exploit this tag as a handle for selective drug delivery to labeled cells.

3.2 Biosynthetic Route to Track Incorporation of Precursor Molecules

Complex processes including lipid biosynthesis and vesicle- and protein-mediated transfer regulate the composition and subcellular localization of signaling lipids, and defects in any of these processes typically result in disease. Several routes are well known for the biosynthesis of PS, essentially depending on the nature of organism, although many of these processes exploit serine as a substrate for an exchange reaction with a lipid (Figure 3.1). For example, prokaryotes commonly convert cytidine diphosphate-diacylglycerol (CDP-DAG) and serine to PS, while mammalian cells (Figure 3.2) instead utilize a transphosphatidylation reaction with phosphatidylcholine (PC) or phosphatidylethanolamine (PE). The biosynthetic route below shows a partial yeast phospholipid production starting form glycerol 3-phosphate (G3P), which is biocatalyzed by acyl-coenzyme A (Acyl-CoA) and forms lyso-phosphatidic acid (LPA) and then phosphatidic acid (PA) in two consecutive reactions. In the next phase, PA is dephosphorylated to diacylglycerol (DAG) or converted to
cytidine diphosphate- (CDP-) DAG, which readily transforms into PS through reaction with serine. Deacylation of PS results in lyso-phosphatidylserine (LPS). Alternatively, decarboxylation of PS may occur to generate phosphatidylethanolamine (PE). PE can then undergo three methylations, progressing through monomethylphosphatidylethanolamine (MMPE) and dimethylphosphatidylethanolamine (DMPE) to ultimately form phosphatidylcholine (PC). Alternatively, PE and PC can be converted back to PA, and DAG can instead be converted to PE and PC through reaction with CDP-ethanolamine (CDP-ETA) and CDP-choline, respectively.

Figure 3.1: Lipid biosynthetic pathway in *Saccharomyces cerevisiae* showing production of different phospholipids and their interconversions.
Figure 3.2: Lipid biosynthetic pathway for mammalian cells showing various phospholipids generation from glycerol-3 phosphate.

3.3 Objectives of Labeling Phospholipids

Metabolic labeling studies are immensely useful as they enable the dynamic detection and characterization of complex biomolecules in cells. Dramatic advancements in labeling have been achieved by introducing diminutive clickable tags onto substrates, providing analogs that successfully infiltrate biosynthetic pathways and generate functionalized products in cells. Prior work on labeling studies has demonstrated the labeling of complex cell-surface glycans using sugar precursor analogs bearing clickable tags. Later, researchers become more interested in labeling lipid analogs as well through a similar approach. For example, tagged fatty acids have been extensively exploited to study posttranslational lipidation. Phospholipids including PC and glycophospatidylinositol (GPI) anchors have been studied by labeling with tagged choline and inositol analogs, respectively. In 2009 Salic et al.
described a protocol where they demonstrated incorporation of labeled choline precursors into PC.\textsuperscript{142} Next, chemical derivatization was achieved, followed by microscopic visualization to confirm the labeling.

The present study uses this strategy to biosynthetically label and microscopically image phospholipids within cellular membranes, and particularly PS. As has already been mentioned, since PS is a promising targeting group as a biomarker, our aim is to perform the metabolic labeling of PS and other lipids using clickable serine analogs to enhance the ability to track the biosynthesis and localization of PS. Additionally, our labeling study will enable the localization of clickable tags on the outer surfaces of cells undergoing apoptosis and those associated with certain cancers, and thus can provide a handle for delivery and imaging applications.

3.4 Design of Serine Analogs for Metabolic Labeling of PS

In a first step towards these goals, we designed and synthesized serine precursors, one with an azido group and another with an alkyne tag, both for click chemistry derivatization, attached at the amino group of serine (Scheme 3.1). This was designed to achieve the metabolic incorporation of these probes to produce labeled PS products through reaction with CDP-DAG, as shown in biosynthetic pathway (Figure 3.1). For detection of labeled products, we utilized the strain promoted azide-alkyne cycloaddition reaction (SPAAC) to click on a fluorophore, which enabled visualization using confocal microscopy. In addition, mass spectrometry studies were run to detect labeled lipid products from cells in
collaboration with Abigail T. Farmer, a graduate student in the lab of Dr. Shawn Campagna in the Chemistry Department at the University of Tennessee using an Orbitrap Exactive MSs and/or a TSQ Quantum Triple Quadrupole MS.

Scheme 3.1: Strategy for analyzing metabolic labeling of lipids using serine analogs 3.1-3.2 in eukaryotic cells through fluorescence microscopy and mass spectrometry.

3.5 Synthesis of Precursor Serine Analogs

We first designed the reporter-tagged serine analogs, which would be the most suitable and efficient for PS production with minimal modifications. In PS, the head group is attached via the side chain hydroxyl group of serine, and thus either the carboxyl or amine moiety could be utilized for the introduction of clickable tags. Initially, we selected the amine group for modification to preserve
the natural charge of PS. Moreover, modification of the amino group is also beneficial since it discourages the incorporation of serine analog into protein biosynthesis.

Compounds 3.1 and 3.2, containing either a propargyl or azidopropyl moiety attached at nitrogen, were synthesized from \(O\text{-tert-butyl-L-serine tert-butyl ester}\) 3.3 as shown in Scheme 3.1. This was performed through alkylation of protected serine 3.3 with either a propargyl group to 3.4 or azidopropyl moiety to 3.5, followed by deprotection to 3.1-3.2, respectively.

Scheme 3.2: Synthesis of serine analogs in two steps.
In the first step, the desired tag (propargyl or propylazido) was installed at onto the amino group, which was followed by deprotection to obtain compounds 3.1 and 3.2.

3.6 Effect of Probes on Growth Curve

We initially set out to determine whether probes had a deleterious effect on cell growth. In doing so, we first pursued the growth curve with yeast cells. \(S.\) \textit{cerevisiae} cells possess significant similarities in terms of the genes, enzymes
and pathways associated with lipid metabolism in mammalian cells,\textsuperscript{146} and moreover this system was selected for convenient access to an abundance of cells. Therefore, we considered \textit{S. cerevisiae} cells as our model eukaryotic cells for growth curve and mass spectrometry studies. Cells were grown in the presence or absence of azide-tagged serine probe 3.2 (1.5 mM) and standard procedures were used to track optical density over time during cell culture. As can be seen in \textbf{Figure 3.3}, the two resulting growth curves were virtually indistinguishable, indicating that probe 3.2 does not cause cell toxicity. Both growth curves consist of comparable lag phase, log phase and stationary phase. The cell growth in both cases was exponentially accelerating for the first 24 hours and then reached persistent OD600 values, indicating the population remained constant in the stationary phase.

\begin{center}
\textbf{Figure 3.3: Growth curves of yeast cells in the presence (study) or absence (control) of 1.5 mM of serine azide probe 3.2.}
\end{center}
3.7 Mass Spectrometry Results

Our next experiment was designed to assess the efficacy of these probes for the infiltration of biosynthetic pathways in live cells to produce labeled lipid products (Scheme 3.1A). For this, we performed mass spectrometry-based lipidomics to detect lipid products bearing the added clickable handle. Cells were grown in the presence and absence of tagged serine analogs, lysed, and then subjected to LC-MS/MS to detect labeled products. In studies with alkynyl probe 3.1, we have detected labeled products corresponding to PS, LPS, and PC (Table 3.1, experimental section). Based on the Figure 3.1 phospholipid chemical transformations, all these lipid products are highly reasonable to include the added click tag. Probe 3.2 delivered similar results as azide-tagged products corresponding to PS, PE, DMPE, PC, and LPS were detected (Table 3.2, experimental section). These results provide evidence that serine probes 3.1-2 are effective as substrates for conversion to PS, after which they can be further decarboxylated to PE derivatives and then undergo methylation to PC structures, passing through DMPE intermediates. PS products can also undergo hydrolysis to LPS. Importantly, these data validate that incorporation of the clickable tags at nitrogen does not deter the normal entry into multiple lipid biosynthetic pathways. However, it should be noted that for mass spectrometry results with 3.2, we also detected peaks corresponding to labeled lipids in one of the two controls, which is concerning potential issue. At this point, we are trying to rerun this experiment.
Additionally, in studies using probe 3.1, we observed low levels of endogenous lipids in the controls, which we are currently investigating as well.

In addition to yeast, we have attempted different cell lines to acquire mass spectrometry data for labeled phospholipids. For example, T24 (human bladder cancer cell) and cho1 mutant yeast cell lines were run to detect labeled phospholipids. The cho1 mutant cell line lacks the PS synthase enzyme, and thus is unable to convert serine analogs into labeled phospholipids, and can be considered as negative control. The T24 cell line was pursued to perform studies in human cells, but we didn’t see any labeled species and the cho1 mutant didn’t show us any difference from wild type yeast cells. One of the reasons might be the extraction procedure we used for cell lysis and extraction of lipids. Here, we used hydrochloric acid (0.1N in methanol) for lipid extraction from membranes which might cause hydrolysis of lipid head group. To solve this problem, we modified our lipid extraction procedure, ultimately using a modification of Bligh and Dyer method. Moreover, different concentrations of probes have been used in metabolic labeling of phospholipids in live cells. It has been found that the mean concentration of serine in yeast cells is around 1.2 mM and the same concentration for probe 3.2 was applied for growing yeast cells.

3.8 Fluorescence Imaging of Lipid Labeling in Cells

The next experiment we performed utilized fluorescence microscopy to exploit the clickable tags of labeled phospholipids and click chemistry to visualize the localization of labeled lipids in cells (Scheme 3.1B). For this study, we chose N-
azidopropyl serine probe 3.2, which allowed for labeling through SPAAC (copper-free click chemistry) using dibenzocyclooctyne-Cy3 (DBCO-Cy3) reagent 3.6. These studies were performed using larger mammalian cells, specifically T24 bladder cancer cells, to enhance the size and resolution of images. In this experiment, T24 cells were incubated with and without the probe 3.2 (100 µM) overnight. Next, they were washed with PBS++ buffer and incubated with 3.6 (1 µM) for one hour. After incubation, the cells were again washed with PBS++ buffer to remove unbound dye from the cell culture. Using 4% paraformaldehyde and ProLong Diamond Antifade Mountant with DAPI the cells were fixed and stained. After drying, the coverslips bearing the cells were sealed and subjected to fluorescence microscopy. The results revealed that cells treated with probe 3.2 provided a strong fluorescence signal (Figure 3.4A), while untreated cells yielded minimal background (Figure 3.4C). Furthermore, the fluorescence was primarily localized at membrane portions within the cells, which supports the incorporation of the label into lipid products. These results further validate the labeling of lipid products and demonstrate the ability to derivatize resulting compounds with fluorophores for their detection via microscopy.

3.9 Fluorescence Imaging of Liposome Targeting of PS in Cells

After performing successful experiments using fluorescence labeling and microscopy, we designed an experiment to exploit the lipids labeled with clickable tags to demonstrate delivery based on the presence of click tag towards eventual selective drug delivery.
Figure 3.4: Fluorescence microscopy images of tagged products resulting from azido-serine probe in human T24 cells.

A. Representative fluorescent image showing that cells treated with 3.2 show significant fluorescence attributed to the labeling of lipid products. B. Bright field image of treated cells. C. Control cells not treated with 3.2 show minimal fluorescence. D. Bright field image of control. Scale bar equals 50 µm.
As has already been mentioned, due to the translocation of PS to membrane outer leaflets driven by apoptosis, PS presence on cell surfaces could provide a marker for the targeting of certain cancers. **Scheme 3.3** depicts our initial strategy for evaluating the prospects of this delivery system by targeting liposomes containing cyclooctyne-lipid 3.7 to cells bearing labeled PS. This was initially investigated through the delivery of fluorescent markers by incorporating commercially available fluorescent lipids into the delivery liposomes. First, compound 3.2 was fed to T24 cells for conversion into tagged lipids. These cells were cultured at Barrera’s lab, and the cell growth in presence of probe was performed by Daiane S. Alves and Nicholas M. Wadsworth, an undergraduate student working for Dr. Francisco Barrera. Next, the cell samples were washed with the PBS++ buffer (supplemented with 1 mM MgCl₂ and 100 µM CaCl₂). Study samples were then incubated with liposomes primarily composed of PC (89.9%) as the bulk lipid along with DSPE-PEG(2000)-DBCO (3.7, 5%) for selective reaction with the azide tag of 3.2 via SPAAC (**Scheme 3.3**). Additionally, PEG(2000)-PE amine (5%) was included, which is commonly used to shield liposomes from decomposition during drug delivery applications. Finally, rhodamine-labeled PE (0.08%) was included to enable detection of liposome localization through fluorescence microscopy. In control liposomes, 3.7 was excluded and replaced with additional PC. After washes with PBS++ buffer (supplemented with 1 mM MgCl₂ and 100 µM CaCl₂), fluorescence microscopy experiments were performed.
Scheme 3.3: Cartoon description of targeted delivery of fluorescent liposomes to cells through SPAAC driven by the presence of clickable lipids on the cell surface.

Representative microscopy data show that treatment of cells with probe 3.2 and liposomes containing 3.7 led to significant delivery of fluorescent lipids to cells (Figure 3.5A), which did not occur when the same labeled cells were treated with liposomes lacking 3.7 (Figure 3.5C). We have attempted this experiment with various liposomes compositions such as the exclusion of PEG(2000)-PE and using 2% Rhodamine PE. The best results we acquired when we incorporated 5% PEG(2000)-PE and 0.08% rhodamine PE in liposomes. We also tried different incubation times, 6, 16 and 24 hours, of which 16 hours appeared to provide the optimal results.
Figure 3.5: Fluorescence microscopy images of tagged products resulting from azido-serine probe in human cells through labeling with fluorescent reagent.

A. Representative fluorescent image shows that cells treated with 3.2 and fluorescent liposomes containing 3.7 yield signal B. DAPI nuclear stain in the second channel of treated cells. C. Control cells treated with 3.2 and liposomes lacking 3.7 show diminished fluorescence. D. DAPI nuclear staining of control. Scale bar corresponds to 25 µm.
3.10 Imaging Studies with Other Serine Probes

Another serine analog, C-alkynyl-serine probe 3.8, was synthesized for the labeling and mass spectrometry experiments. The synthesis of 3.8 (Scheme 3.4) began with 3.9 that has free hydroxyl and carboylic acid groups. In the first step, the hydroxyl group was protected as a tetrahydropyran (THP) group, and next the propargyl group was installed via peptide coupling reaction conditions. Finally, the boc and THP groups were deprotected using TFA in DCM.

Scheme 3.4: Synthesis of C-alkynyl probe 3.8 from Boc-protected L-serine.

For fluorescence labeling and imaging studies with this serine probe, we employed copper catalyzed click chemistry using azide-Fluor 488 (2.14) alongside probe 3.2. For this, we grew yeast cells with and without compounds 3.2 and 3.8 (100 micromolar) individually. Next, cells were washed with PBS buffer, and treated with azide-Fluor 488 (2.14) (10 micromolar) and lastly, samples were washed three times with 20% DMSO-water solution.
Figure 3.6: Fluorescence images for *S. cerevisiae* cells with N-alkynyl and C-alkynyl serine probes. A, B and C are images for cells grown without any probe compound, and in the presence of 3.1 and 3.8, respectively. D, E and F are bright field images of the corresponding samples.
As Figure 3.6 indicates, the control that has no probe (neither 3.1 nor 3.8) does not show much fluorescence, whereas the other two study samples yield significant fluorescent in yeast cells. While fluorescence image for the study (treated with 3.8) is significantly greater than the control (not treated with 3.8), the fluorescence does not appear to be localized across membranes within the cells. Moreover, with C alkynyl probe 3.8, we didn’t detect a number of phospholipids on mass spectrometry because the tag on the C-terminus limits its conversion from PS into other phospholipids. However, this could be a positive for achieving specificity for the labeling of PS in cells.

3.11 Tagged Analogs of LPA and LPS for Lipid Labeling

An alternate strategy we have begun pursuing for the labeling of lipids involves the use of tagged lyso-lipids. As previously discussed, lipids including LPA and LPS act as substrates that are converted to the important phospholipids PA and PS, respectively, through acylation. We have designed labeled LPA (3.21) and LPS (3.24) probes that contain an azido tag appended at the end of the lipid tail to further separate the label from the lipid head group, which is the primary site for lipid modification and recognition. The azido-tail is attached by an ether linkage to avoid release of the clickable label by acyl chain hydrolysis.

Initial synthetic progress towards these probes is shown in Scheme 3.5. This route starts with the protection of a (S)-glycerol acetonide (2.9) with a p-methoxybenzyl (PMB) group and then the acetonide ring was deprotected using acid to 3.13. Next, alkylation with 1, 11-dibromoundecane in the presence of
butyl tin(II) oxide was used to obtain 3.14, after which the deprotection of the PMB group using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in DCM-H$_2$O (18:2) would give us 3.15. Different ratio of DCM-H$_2$O solutions were tried that resulted in very poor yield of 3.15. Once synthesized, compound 3.15 could be converted into 3.16 by treatment with sodium azide in DMF.

Scheme 3.5: Synthesis of azide tagged tail for LPA and LPS.

We next pursued an alternate route, shown in Scheme 3.6, using S-glycidol (3.17) as the starting material. This was treated with boron trifluoride-diethyletherate in the presence of 11-bromo-1-undecanol (3.18) to produce 3.15. Next, bromo group was replaced with azido group using sodium azide, followed by a phosphorus coupling reaction with diethyl chlorophosphate (3.19) in the presence of tetramethyl piperidine (TMP) to obtain 3.20. Finally, deprotection with trimethylsilylbromide (TMSBr) in DCM and methanol gave us LPA 3.21.
Scheme 3.6: Synthesis of LPA with an azide tagged acyl tail.
Scheme 3.7: Synthesis of LPS with an azide tagged acyl tail.

LPS probe 3.24 could also be accessed using 3.16 as shown in Scheme 3.7. This can be reacted with ethyl dichlorophosphate in the presence of TMP, followed by a second coupling reaction with protected serine 3.22 in the presence of TMP to provide 3.23. Deprotection of the Boc and ethyl groups can be carried out by treatment with TFA and TMSBr in DCM, respectively, to produce 3.24.

3.12 Conclusion

We demonstrated that serine analogs with click tags can be incorporated in eukaryotic and prokaryotic cells, which was validated by fluorescence imaging and mass spectrometry. The fluorescence imaging results show labeling only in the presence of tagged serine probes, while the mass spectrometry results indicate promising peaks for labeled phospholipids that are being followed with further controls. The labeled PS has also been utilized as a handle for liposomal
delivery, which has been validated by the delivery of fluorescence-tagged clickable liposomes. Future studies will involve the synthesis of LPS and incorporation of LPA and LPS as an alternate avenue for PS labeling. Since the two compounds have click tags at the end of the acyl tail, this reporter group will remain unperturbed when these probes undergo metabolic interconversions to other phospholipids, and could therefore be useful for labeling additional compounds. We will additionally further study serine probe 3.8 in which the click tag is introduced as an amide in place of the carboxylic acid as a means for avoiding decarboxylation in the labeling of PE and downstream lipids. The trafficking of lipids into different cellular components can be tracked down using mutant cell lines. For instance, the trafficking of PS to mitochondria for decarboxylation could be studied using a psd1Δ mutant, which lacks the mitochondrial PS decarboxylase. We can further analyze the trafficking of PS to endosomes by blocking PS to PE synthesis with the psd2Δ mutant. We can additionally analyze PE localization using pem1Δ pem2Δ mutants to block methylation.

3.13 Experimental

Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. L-α-Phosphatidylcholine (mixed isomers from chicken egg), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzocyclooctyl(polyethylene glycol)-2000] (ammonium salt), (DSPE-PEG(2000)-DBCO), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-
[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000) amine), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-PE) were purchased from Avanti Polar Lipids, Inc. O-tert-Butyl-L-serine tert-butyl ester hydrochloride (3.3), propargyl bromide, and 1,3-dibromopropane were purchased from Fisher Scientific. Dibenzocyclooctyne-cyanine 3(DBCO-Cy3) was obtained from Sigma Aldrich. (S)-(−)-glycidol, diethyl chlorophosphate, ethyl dichlorophosphate N-(tert-butoxycarbonyl)-L-serine were purchased from Fisher Scientific. SiliaPrep C18 (17%, 2g, 6mL, 40-63um) was provided by SiliCycle Inc. Dry solvents were obtained from a Pure solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian Mercury 500 and 600 spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF mass spectrometer with high-resolution capabilities spectrometer. Liposome extruder and polycarbonate membranes were obtained from Avestin (Ottawa, Canada). Ultrapure water was purified via a Millipore water system (≥ 18 MΩ·cm triple water purification system). Solution fluorescence studies were performed using a Perkin Elmer LS55 fluorimeter. Cell images were captured using a Leica SP8 White Light Laser Confocal microscope system using a 63x 1.4na oil objective.

**Liposome preparation.** Stock solutions were made of rhodamine-PE, DSPE-PEG(2000)-DBCO (3.7), DSPE-PEG(2000) amine, and PC to make liposomes.
Examples of each stock solution used are as follows: 1.0 mg rhodamine-PE, 50.1 mg PC, 5.0 mg DSPE-PEG(2000)-DBCO (3.7) and 2.5 mg DSPE-PEG(2000) amine were each weighed into separate vials and dissolved in 1000 µL chloroform to form stock solutions of 0.76 mM rhodamine-PE, 65.05 mM PC, 1.62 mM DSPE-PEG(2000)-DBCO (3.7) and 0.89 mM DSPE-PEG(2000) amine. Using these stock solutions, 1.80 µL rhodamine-PE stock (0.08%), 54.0 µL DSPE-PEG(2000)-DBCO (3.7, 5%), 98.2 µL DSPE-PEG(2000) amine stock (5%), and 24.2 µL PC stock (89%), were combined in a glass vial. For control liposomes, 1.80 µL rhodamine-PE stock (0.08%), 98.2 µL DSPE-PEG(2000) amine stock (5%), and 25.5 µL of PC stock (94.9%) were combined in a second vial. In each case, the chloroform solvent was removed under a stream of nitrogen and the resulting mixture were placed under vacuum overnight. The dried lipids were then hydrated by adding 350 µL of Milli-Q water, and rotated on a rotary evaporator for 60 min at 60 °C. The liposomes were then subjected to 10 freeze-thaw cycles and extruded (21 times) to uniform size and lamellarity using an extruder containing a 200 nm polycarbonate filter.

Fluorescence imaging of lipid labeling in cells and delivery of liposomes.
T24 cells (transitional cell bladder carcinoma, ATCC® HTB-4™) were cultured in a humidified incubator at 37°C under 5% CO₂ in McCoy medium (Invitrogen) supplemented with 10% of fetal bovine serum (Invitrogen). Briefly, cells were seeded (10⁵ cells per well) in a 12-well plate 2 days prior to the experiments to cover slips, treated with or without 1 mM of 3.2, and incubated for 16h in Opti-
MEM medium (Invitrogen). Cells were next washed with PBS++ buffer (supplemented with 1 mM MgCl₂ and 100 µM CaCl₂). For experiments involving the fluorescence labeling of lipids (Figure 1), cells grown in the presence or absence of 3.2. were next incubated for 1 h with 98 µL of a 1.02 mM stock of DBCO-Cy3 dye 3.6 in a total volume of 1 mL of PBS++. For liposome delivery studies, all cells were grown with 3.2, after which they were treated for 1 h with liposomes (5 mM) either containing or lacking 3.7 prepared as described in the previous section. Finally, cells were washed four times with PBS++, fixed for 30 min in 4% paraformaldehyde and mounted with ProLong Diamond Antifade Mountant with DAPI (Invitrogen) or Vectashield Antifade Mounting Medium (Vector Laboratories). Cells were visualized employing a Leica SP8 White Light Laser Confocal microscope. Contrast and brightness settings were selected so that all pixels were in the linear range. Images are the product of fourfold line averaging.

**Yeast cell growth and mass spectrometry procedures**

*Saccharomyces cerevisiae* cell growth. *S. cerevisiae* TRY 181 (wild-type, uraΔhisΔ) strained cells was streaked or spread on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plates from inoculum and incubated at 30 °C, and single colonies were seen after ~24h. Next, from a single colony, a loop of cells was added to 5 mL 2% galactose minimal medium (with galactose as a carbon source) into a glass cell culture tube and incubated at 30 °C at 225 rpm with good aeration overnight to an OD600 (optical density) of 2.33. Next, 214.6-
μL aliquots from this culture were transferred into two detergent-free glass cell culture tubes containing 4.78 mL of fresh 2% galactose medium with and without N-propylazide serine analog 3.2 (300 μL of a 25 mM solution), both of which were incubated for 16 to 17 h until the OD600 value reached ~1.0. For good aeration, the medium constituted no more than one-fifth of the total tube volume, and growth was carried out in a shaking incubator at 225 rpm. Thereafter, the cell solution was collected in 10 mL conical vial and centrifuged at 3000 x g for 5 min, followed by wash with 5 mL of cold water and centrifuged again at 3000 x g for 5 min and stored at – 80 °C for mass spectrometry lipidomics studies.

**Fluorescence Imaging using Saccharomyces cerevisiae cells using copper-catalyzed click reaction.** Two 1 mL samples of S. cerevisiae cells, one treated with compound 3.1 (study) and one untreated (control), were incubated with 10 μM Azide-Fluor 488 dimethylsulfoxide (DMSO) solution for 1 hr at rt. For this, first the Azide-Fluor 488 (2.60 μL, 3.828 mM) DMSO solution, copper (II) sulfate pentahydrate (4.50 μL, 4.01 mM) followed by Tris(3-hydroxypropyltriazolylmethyl)amine (13.30 μL, 8.29 mM), sodium ascorbate (3.10 μL, 325 mM) and 976.5 μL mili Q water were added. Next, the cell samples were centrifuged for 3 min at 2500 rpm, the dye solutions were removed, and the cells were washed with 20% DMSO in water three times, vortexing and centrifugation at 2500 rpm for 3 min performed in between each washing step. Next, 1 mL ice-cold methanol was added to each tube, and after incubating for 10 min at 0 °C, the sample was centrifuged. Next, 1 mL of Phosphate Buffered Saline solution
(PBS, pH 7.4, 1.19 mM phosphates) was used to wash the fixed cell samples, which were then centrifuged at 2500 rpm for 3 min. Cells were then subjected to fluorescence microscopy.

**Lipid extraction and mass spectrometry.** This protocol was adapted from that described by Bligh and Dyer. All operations with extracted lipids were done using glass pipettes or syringes; plastics will create a large background signal if they come into contact with chloroform on mass spectra. Frozen cells were resuspended in 1.6 mL of ice-cold distilled water and transferred to high-speed glass centrifuge tubes. 6 mL of chloroform and MeOH (1:2) mix and 0.8 ml of glass beads were added to the cell suspension and vortexed with glass beads two times for 1 min. Next, 2 ml of chloroform was added and mixed gently, later incubated for 5 min at room temperature with occasional mixing. 2 ml of distilled water was added and incubated for 5 min at room temperature with gentle mixing. The entire liquid phase was collected into a new high-speed glass centrifuge tube after centrifuging for 5 min at 3000 x g at room temperature. 3.2 mL of chloroform was added to the remaining cell pellets and vortexed two times for 1 min. The cell pellets were centrifuge for 5 min at 3000 x g at room temperature and the supernatant was transferred to the liquid phase collected before. Now, the whole liquid phase (collected from 2 extraction steps) was centrifuged for 5 min at 3000 x g at room temperature. The upper (aqueous) phase was discarded and the lower (organic) phase was transferred into a new high-speed glass centrifuge tube. To remove residual cell pellets, once again the
organic phase was centrifuged for 5 min at 3000 x g at room temperature. Next, the entire supernatant was transferred into a glass vial and dried under nitrogen. The dried lipid film was then dissolved in 150 μL of methanol: chloroform (9:1) and separated on a Kinetex HILIC column (150 mmx2.1 mm, 2.6 μm) (Phenomenex, Torrance, CA, USA) connected to an Ultimate 3000 autosampler and UHPLC pump and an Exactive benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an electrospray ionization (ESI) probe. Separations were run for 35 min with mobile phase A and B consisting of 10 mM aqueous ammonium formate pH 3 and 10 mM ammonium formate pH 3 in 93% (v/v) acetonitrile (ACN) respectively. The gradient started at 100% B for 1 min; and was from 1 to 15 min decreased to 81% B, from 15 to 15.1 min to 48% B, from 15.1 to 25 min maintained at 48% B, from 25 to 25.1 min increased to 100% B, and from 25.1 to 35 min maintained at 100% B. The column oven temperature was maintained at 25 °C and the temperature of the auto sampler was set to 4 °C. The same LC conditions and buffers were used for all MS experiments with a flow rate of 0.2 mL/min. The spray voltage was set to 4 kV and the heated capillary was set at 350 °C. The sheath gas flow was set to 25 units and the auxiliary gas set to 10 units. These conditions were held constant for both positive and negative ionization mode acquisitions. External mass calibration was performed using the standard calibration mixture and protocol from ThermoFisher approximately every 2 days. For full scan profiling experiments, the MS was run with resolution of 140,000k with a scan range of
100-1500 m/z. Each sample was run in both positive and negative mode. Samples were run in positive and negative mode and full scan settings were the same. For the all ion fragmentation scans, the resolution was 140,000 with a scan range of 100-1500 m/z. The normalized collision energy was 30eV with stepped collision energy of 50%. Full scan data was evaluated using Maven software. Lipid classes were identified by their fragments using Xcalibur software (Thermo Fisher Scientific, San Jose, CA).

\[
\begin{align*}
\text{3.3} & \xrightarrow{1) K_2CO_3} \xrightarrow{2) Br} \text{3.4} \\
1) & \text{K}_2\text{CO}_3 \\
2) & \text{DMF} \quad 35\
\end{align*}
\]

**tert-Butyl 3-(tert-butoxy)-2-(prop-2-yn-1-ylamino)propanoate (3.4).** O-tert-Butyl-L-serine tert-butyl ester hydrochloride (3.3, 0.200 g, 0.788 mmol) was washed with 5% ammonia solution and extracted with 75 mL dichloromethane (DCM) (3 x 25 mL). The DCM layers were collected in a 250 mL round-bottomed flask (RBF), the solvent was removed by rotary evaporation, and the sample was subjected to high vacuum. Next, dry \(N,N\)-dimethylformamide (DMF, 200 mL) was added, followed by anhydrous potassium carbonate (0.239 g, 1.734 mmol) and the mixture was allowed to stir for 30 min. Next, propargyl bromide (0.947 g, 0.946 mmol,) was added. The reaction was then allowed to stir overnight, after which ice-cold water (200 mL) was added, and the aqueous portion was
extracted with dichloromethane (3 x 25 mL). The organic layers were then combined and dried with magnesium sulfate, filtered and concentrated by rotary evaporation. Column chromatography using gradient elution with 10-50% ethyl acetate-hexanes gave 3.4 as yellow oil (72.7 mg, 36% yield). ¹H NMR (500 MHz, CD₃OD): δ 3.6 (dd, J = 8.5 hz, 5.0 hz 1H), 3.54 (m, 2H), 3.42 (d, J = 2.5 Hz, 1H), 3.46 (m, 1H), 2.19 (t, J = 2.5 Hz, 1H), 2.06 (broad s, 1H), 1.47 (s, 9H), 1.15 (s, 9H). ¹³C NMR (126 MHz, CD₃OD): δ 171.80, 81.74, 81.15, 72.93, 71.44, 63.12, 60.65, 36.69, 28.11, 27.37. HRMS-DART [M+H]- calcd for C₁₄H₂₉N₄O₃⁺, 256.1907; found 256.1908.

3-Hydroxy-2-(prop-2-yn-1-ylammonium)propanoic acid trifluoroacetate (3.1). Protected alkynyl-serine 3.4 (25.0 mg, 0.098 mmol) was dissolved in DCM (1 mL), treated with trifluoroacetic acid (TFA, 4 mL), and the solution was allowed to stir for 8 h. The excess TFA was then removed by co-evaporation with DCM and the crude was placed on the high vacuum for overnight. The product 3.1 was purified by reverse phase column chromatography and obtained as a transparent solid (21.4 mg, 85% yield). ¹H NMR (500 MHz, CD₃OD): δ 3.91 (dd, J = 4.1 hz,
2.2 Hz, 2H), 3.90 (dd, J = 2.5 Hz, 1.3 Hz, 2H), 3.84 (t, J = 4.1 Hz, 1H), 2.87 (t, J =
2.6 hz, 1H). \(^{13}\)C NMR (126 MHz, CD\(_3\)OD): \(\delta\) 170.78, 78.06, 73.00, 62.04, 59.02,
35.33. HRMS-DART [M+H]- calcd for C\(_6\)H\(_{10}\)NO\(_3\)^+, 144.0655; found 144.0655.

**1-Azido-3-bromopropane.** 1,3-dibromopropane (0.400 g, 1.98 mmol) was
transferred to a 250 mL RBF in DMF and refluxed with sodium azide (0.155 g,
2.377 mmol) at 55 °C overnight. Next, ice cold water (200 mL) was added and
the crude was extracted with DCM (3 x 25 mL). The remaining water was
adsorbed on anhydrous magnesium sulfate and the solvent was removed by
rotary evaporation to yield light yellow oil (276 mg). The mass spectrum showed
that conversion of bromide to azide group was completed, and the crude was
passed on without any purification.

\[
\begin{align*}
\text{O-tert-Butyl-L-serine tert-butyl ester hydrochloride (3.3), 0.200 g, 0.788 mmol} & \text{ was washed with 5% ammonia solution and extracted with DCM (3 x 25 mL). The DCM layers were combined in a 250 mL RBF, the solvent was removed by rotary evaporation, and the contents were place under high vacuum. Next,}
\end{align*}
\]
200 mL of dry DMF was added, followed by anhydrous potassium carbonate (0.239 g, 1.734 mmol), and the mixture was allowed to stir for 30 min. Next, 1-azido-3-bromopropane (0.155 g, 0.946 mmol) was added. The reaction was then allowed to stir overnight at 54 °C, after which ice-cold water (200 mL) was added, and the aqueous portion was extracted with DCM (3 x 25 mL). The organic layers were then combined and dried with magnesium sulfate, filtered and concentrated by rotary evaporation. Column chromatography using gradient elution with 10-50% ethyl acetate-hexanes gave 3.5 as yellow oil (82.8 mg, 35% yield). $^1$H NMR (500 MHz, CD$_3$OD): δ 5.50 (d, J = 8.5 Hz, 1H), 4.32 (d, J = 8.9 Hz, 1H), 4.15 (m, 2H), 3.78 (m, 1H), 3.53 (d, J = 11.3 Hz, 2H), 3.38 (dd, J = 15.2 Hz, 6.8 Hz, 2H), 1.9 (dq, J = 12.9 Hz, 6.5 Hz, 2H), 1.47 (s, 9H), 1.15 (s, 9H). $^{13}$C NMR (126 MHz, CD$_3$OD): δ 169.57, 81.78, 73.05, 62.25, 61.83, 54.84, 48.17, 28.56, 28.00, 27.30. HRMS-DART [M-H]$^-\text{calcd for } C_{14}H_{29}N_4O_3^+$, 301.2240; found 301.2237.

Protected azido-serine 3.5 (25.0 mg, 0.083 mmol) was dissolved in 1,4-dioxane.
(1.5 mL) and treated with hydrochloric acid (3.5 mL, 8 M), and the mixture was allowed to stir for 8h at 70 °C. After that, the solvent was removed by rotary evaporation, and a reverse phase C18 column provided 3.2 (14.3 mg, 91% yield) as a light yellowish liquid. \(^1\)H NMR (500 MHz, CD\(_3\)OD): \(\delta\) 4.71 (m, 1H), 4.33 (m, 2H), 4.03 (m, 2H), 3.52 (q, \(J = 6\) Hz, 1H), 2.13 (m, 2H). \(^{13}\)C NMR (126 MHz, CD\(_3\)OD): \(\delta\) 169.68, 81.77, 54.86, 29.17, 28.04, 27.33. HRMS-DART [M+H]- calcd for C\(_6\)H\(_{11}\)N\(_2\)O\(_2\)^+, 143.082; found 143.082.

(2S)-2-((tert-butoxycarbonyl)amino)-3-((tetrahydro-2H-pyran-2-yl)oxy)propanoic acid (3.10). \(N\)-(tert-Butoxycarbonyl)-L-serine (3.9, 0.500 g, 2.44 mmol) was transferred to a 250 mL round-bottomed flask and added pyridinium \(p\)-toluene sulfonate (0.736 g, 2.92 mmol) and dihydropyran (0.267 g, 2.92 mmol) in 150 mL DCM. The reaction mixture is stirred for overnight and extracted with DCM (3 x 25 mL). The DCM layers were collected in a 250 mL round-bottomed flask (RBF) and the solvent was dried with magnesium sulfate, filtered and removed by rotary evaporation. Column chromatography using gradient elution with 10-50% ethyl acetate-hexanes gave 3.10 as white solid (0.663 mg). It was used in the next step without characterization.
**tert-butyl ((2S)-1-oxo-1-(prop-2-yn-1-ylamino)-3-((tetrahydro-2H-pyran-2-yl)oxy)propan-2-yl)carbamate (3.11):** To a 250 mL round-bottomed flask, 3.10 (0.325 g, 1.12 mmol) was taken with hydroxybenzotriazole (HOBt, 0.151 g, 1.12 mmol) and \( O\)-(benzotriazol-1-yl)-\(N,N,N',N'\)-tetramethyluronium hexafluorophosphate (HBTU, 0.425 g, 1.12 mmol) and DIEA (0.434 g, 3.36 mmol). After stirring for 15 min, propargylamine (0.74 g, 1.347 mmol) was added to the reaction mixture which was allowed to react for overnight. After reaction completion, the reaction mixture was extracted with DCM (3 x 25 mL). The DCM layers were collected in a 250 mL round-bottomed flask (RBF), the solvent was dried with magnesium sulfate, filtered and removed by rotary evaporation. Column chromatography using gradient elution with 10-50% ethyl acetate-hexanes gave 3.11 as yellow oil (0.139 mg).
(S)-3-hydroxy-1-oxo-1-(prop-2-yn-1-ylamino)propan-2-ammonium 2,2,2-trifluoroacetate (3.8). Compound 3.11 (0.100 g, 0.306 mmol) was dissolved in DCM (5 mL) and TFA (5 mL) and stirred overnight. The excess TFA was evaporated off using rotary evaporation, and a reverse phase C18 column (5% methanol in water) provided 3.8 (60.1 mg, 77% yield) as a light yellowish liquid. 

$^1$H NMR (500 MHz, CD$_3$OD): δ 4.07 (d, J = 2.6 Hz, 1H), 3.99 (d, J = 2.6 Hz, 1H), 3.93 (m, 2H), 3.82 (dd, J = 11.2, 5.8 Hz, 1H), 2.62 (t, J = 2.6 Hz, 1H). $^{13}$C NMR (126 MHz, CD$_3$OD): δ 170.01, 155.43, 99.45, 80.07, 79.34, 71.45, 67.64, 62.60. HRMS-DART [M-H]$^-$ calcd for C$_6$H$_{11}$N$_2$O$_2$$^+$, 143.082; found 14.082.

(S)-3-((11-bromoundecyl)oxy)propane-1,2-diol (3.15). S-glycidol (3.17), 0.500 g, 6.75 mmol) in DCM (250 mL) was transferred to a 500 mL round-bottomed flask and boron trifluordie diethyl etherate (0.96 g, 0.675 mmol) was added to it,
stirred for 15 min. Next, 1-bromoundecanol **3.18** (1.8 g, 7.166 mmol) was added. The reaction mixture is stirred for overnight and extracted with DCM (3 x 50 mL). The DCM layers were collected in a 250 mL round-bottomed flask (RBF), the solvent was dried with magnesium sulfate, filtered and removed by rotary evaporation. Column chromatography using gradient elution with 10-40% ethyl acetate-hexanes gave **3.15** as white solid (0.615 mg). It was used in the next step without characterization.

![Chemical structure](image)

**3.15**

**3.16**

**(S)-3-((11-azidoundecyl)oxy)propane-1,2-diol (3.16).** To a 250 mL round-bottomed flask, **3.15** (0.400 g, 1.229 mmol) transferred in DMF and sodium azide (0.199 g, 3.074 mmol) were added and refluxed at 55 °C overnight. Next, ice cold water (200 mL) was added and the crude was extracted with DCM (3 x 25 mL). The remaining water was adsorbed on anhydrous magnesium sulfate and the
solvent was removed by rotary evaporation to yield light yellow oil (295 mg). The crude was passed on without any purification.

(R)-3-((11-azidoundecyl)oxy)-2-hydroxypropyl diethyl phosphate (3.20). To a 50 mL round-bottomed flask containing 5 mL dry DCM was added 3.19 (0.016 g, 0.090 mmol) and 2,2,6,6-Tetramethylpiperidine (TMP) (0.0127 g, 0.090 mmol) and stirred at 0 °C for 15 min. Then, 3.16 (0.020 g, 0.070 mmol) was added and stirred for overnight. Resulting reaction mixture was treated with aqueous solution and extracted with DCM (3 x 15 mL). Later, DCM layer was dried over magnesium sulfate, filtered and DCM layer was evaporated off using a rotary evaporation. Column chromatography using gradient elution with 25%-100% ethyl acetate-hexanes gave 3.20 as white solid (0.021 g).
(R)-3-((11-azidoundecyl)oxy)-2-hydroxypropyl dihydrogen phosphate (3.21).

In a 25 mL round-bottomed flask, added 3.20 (0.020 g, 0.0472 mmol) and trimethylsilylbromide (TMSBr) (0.145 g, 0.944 mmol) in 2 mL dry DCM. The reaction mixture was allowed to stir for 10-12 h, after that the DCM layer was evaporated off and dry methanol was added and stirred for 2 h. The resulting mixture was again subjected to rotary evaporation and a reverse phase C18 (10% methanol in water) column provided 3.21 (60.1 mg, 77% yield) as a light yellowish liquid. $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 3.66 (m, 1H), 3.54 (m, 2H), 3.34 (m, 2H), 3.27 (t, $J= 2.56$ Hz, 1H), 1.60 (m, 4H), 1.27 (s, 16H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 71.82, 70.41, 64.45, 63.04, 32.77, 29.38, 27.18, 25.70. $^{31}$P NMR (75 MHz, D$_2$O) $\delta$ 0.21. HRMS-DART [M-H] - calcd for C$_{14}$H$_{31}$N$_3$O$_6$P$^+$, 368.1943; found 368.2542.
3.14 List of Spectra

Spectra 3.1: \(^1\)H NMR and \(^{13}\)C NMR of compound 3.4. tert-Butyl 3-(tert-butoxy)-2-(prop-2-yn-1-ylamino)propanoate.
Spectrum 3.2: Mass Spectrum of compound 3.4. tert-Butyl 3-(tert-butoxy)-2-(prop-2-yn-1-ylamino)propanoate.
Spectra 3.3: $^1$H NMR and $^{13}$C NMR of compound 3.1. 3-Hydroxy-2-(prop-2-yn-1-ylammonium)propanoic acid trifluoroacetate.
Spectrum 3.4: Mass Spectrum of compound 3.1. 3-Hydroxy-2-(prop-2-yn-1-ylammonium)propanoic acid trifluoroacetate.
Spectra 3.5: $^1$H NMR and $^{13}$C NMR of compound 3.5. 

tert-butyl-(3-azidopropyl)-O-(tert-butyl)-L-serinate.
Spectrum 3.6: Mass Spectrum of compound 3.5. tert-butyl N-(3-azidopropyl)-O-(tert-butyl)-L-serinate.
Spectra 3.7: $^1$H NMR and $^{13}$C NMR of compound 3.2. 2-((3-azidopropyl)ammonium)-3-hydroxypropanoic acid chloride.
Spectrum 3.8: Mass Spectrum of compound 3.2. 2-((3-azidopropyl)ammonium)-3-hydroxypropanoic acid chloride
Spectra 3.9: $^1$H NMR and $^{13}$C NMR of compound 3.8. (S)-3-hydroxy-1-oxo-1-(prop-2-yn-1-ylamino)propan-2-ammonium 2,2,2-trifluoroacetate.
Spectra 4.1: $^1$H NMR and $^{13}$C NMR of compound 3.21. $(R)$-3-((11-azidoundecyl)oxy)-2-hydroxypropyl dihydrogen phosphate.
Table 3.1: List of alkyne labeled phospholipids detected on mass spectrophotometer.

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Predicted m/z</th>
<th>Observed m/z</th>
<th>Error (ppm)</th>
<th>RT</th>
<th>No. of Carbon</th>
<th>Predicted % 13C peak</th>
<th>% 13C peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab LysoPS 2</td>
<td>C25H44NO9P</td>
<td>532.270</td>
<td>532.266</td>
<td>7.14</td>
<td>12</td>
<td>25</td>
<td>27.50</td>
<td>23.1 7</td>
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<td>Lab LysoPS 3</td>
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<td>560.300</td>
<td>560.298</td>
<td>4.46</td>
<td>12</td>
<td>27</td>
<td>29.70</td>
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<tr>
<td>Lab PC10</td>
<td>C42H80NO8P</td>
<td>758.559</td>
<td>758.569</td>
<td>12.52</td>
<td>11</td>
<td>42</td>
<td>46.20</td>
<td>45.4 6</td>
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<tr>
<td>Lab PC5</td>
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<td>726.507</td>
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<td>9</td>
<td>40</td>
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<tr>
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<td>40</td>
<td>44.00</td>
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<tr>
<td>Lab PS3</td>
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<td>770.497</td>
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<td>9</td>
<td>41</td>
<td>45.10</td>
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<td>796.513</td>
<td>0.38</td>
<td>9</td>
<td>43</td>
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<tr>
<td>Lab PS6</td>
<td>C43H78NO10P</td>
<td>798.529</td>
<td>798.529</td>
<td>0.09</td>
<td>10</td>
<td>43</td>
<td>47.30</td>
<td>44.0 8</td>
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<tr>
<td>Lab PS7</td>
<td>C45H80NO10P</td>
<td>824.545</td>
<td>824.545</td>
<td>0.49</td>
<td>10</td>
<td>45</td>
<td>49.50</td>
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Table 3.2: List of azide labeled phospholipids detected on mass spectrophotometer.

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<th>Name</th>
<th>Formula</th>
<th>Predicted m/z</th>
<th>Observed m/z</th>
<th>Error (ppm)</th>
<th>RT</th>
<th>No. of Carbon</th>
<th>Predicte% 13C peak</th>
<th>% 13C peak</th>
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</thead>
<tbody>
<tr>
<td>Lab PS6</td>
<td>C43H82N4O10P</td>
<td>845.576</td>
<td>845.562</td>
<td>16.90</td>
<td>18</td>
<td>43</td>
<td>47.30</td>
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<tr>
<td>Lab PS4-N2</td>
<td>C41H79N2O10P</td>
<td>790.547</td>
<td>790.558</td>
<td>14.28</td>
<td>14</td>
<td>41</td>
<td>45.10</td>
<td>39.62</td>
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<tr>
<td>Lab PS3</td>
<td>C41H78N4O10P</td>
<td>817.545</td>
<td>817.538</td>
<td>8.23</td>
<td>16</td>
<td>41</td>
<td>45.10</td>
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<tr>
<td>Lab PS1-Na</td>
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<td>783.456</td>
<td>9.75</td>
<td>13</td>
<td>37</td>
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<td>Lab PC12-Na</td>
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<tr>
<td>Lab PC2</td>
<td>C36H72N4O8P</td>
<td>719.508</td>
<td>719.509</td>
<td>1.52</td>
<td>13</td>
<td>36</td>
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<td>Lab MMPE9-Na</td>
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<td>Lab DMPE8-Na</td>
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<td>837.584</td>
<td>837.576</td>
<td>9.27</td>
<td>8</td>
<td>43</td>
<td>47.30</td>
<td>66.37</td>
</tr>
</tbody>
</table>
3.15: List of Chromatogram and Mass Spectra of N-Alkynyl Labeled Phospholipids

Chemical Formula: $C_{25}H_{43}NO_5P^{-}$
Exact Mass: 532.268
LAB LYSOPS3

Chemical Formula: $C_{27}H_{47}NO_9P^-$
Exact Mass: 560.299
LAB PC10

$C_{42}H_{81}NO_8P^+$

Exact Mass: 758.569
LAB PC5

Chemical Formula: \( C_{40}H_{73}NO_8P^+ \)

Exact Mass: 726.507
LAB PC7

Chemical Formula: \( C_{40}H_{75}NO_8P^+ \)

Exact Mass: 730.538
LAB PS3

Chemical Formula: $C_{41}H_{73}NO_{10}P^-$
Exact Mass: 770.498
Chemical Formula: $C_{43}H_{75}NO_{10}P^-$
Exact Mass: 796.513
Chemical Formula: $\text{C}_{43}\text{H}_{77}\text{NO}_{10}\text{P}^-$
Exact Mass: 798.529
Chemical Formula: $\text{C}_{46}\text{H}_{79}\text{NO}_{10}\text{P}^-$

Exact Mass: 824.545
3.16 List of Chromatogram and Mass Spectra of N-Propylazido Labeled Phospholipids

LAB DMPE8-Na

Chemical Formula: $C_{43}H_{83}N_4NaO_8P^+$

Exact Mass: 837.584
LAB LYSOPS1

Chemical Formula: $C_{25}H_{50}N_4O_9P^+$
Exact Mass: 581.331
LAB MMPE2

Chemical Formula: $C_{36}H_{72}N_4O_8P^+$
Exact Mass: 719.508
LAB MMPE9-Na

Chemical Formula: C_{42}H_{81}N_{4}NaO_{8}P^{+}
Exact Mass: 823.568

N_{3} NH_{2}
LAB PC2

Chemical Formula: \( \text{C}_{36}\text{H}_{72}\text{N}_{4}\text{O}_{8}\text{P}^+ \)

Exact Mass: 719.508
LAB PC12-Na

Chemical Formula: $C_{44}H_{85}N_4Na_8O_6P$

Exact Mass: 851.600
LAB PS1-Na

Chemical Formula: C₃₇H₆₉N₄NaO₁₀P⁺
Exact Mass: 783.464
Chemical Formula: $C_{41}H_{79}N_4O_{10}P^+$
Exact Mass: 817.545
LAB PS4-N$_2$

Chemical Formula: C$_{41}$H$_{79}$N$_2$O$_{10}$P$^+$
Exact Mass: 790.547
LAB PS6

Chemical Formula: $C_{43}H_{82}N_4O_{10}P^+$
Exact Mass: 845.576
CHAPTER FOUR
SYNTHESIS OF POTENTIAL PHOSPHATIDYLSERINE SYNTTHASE (PS) INHIBITORS

4.1 Detrimental Effect of Candida Species

Only a relatively small number of Candida species are pathogenic for humans and can cause serious infections in a suitable environment or an immunocompromised body system where the immune system in the host is damaged. Organisms capable of triggering a variety of superficial and deep-seated mycoses generally exist in the gastrointestinal and genitourinary tracts as well as the exteriors of mucosa tissues. Moreover, Candida species (spp.) have the unique ability to form biofilms, a uniform framework of microorganism on the adhering surface. Biofilms grown by Candida spp. on implanted medical devices are recognized as one of the primary source for spreading infections. Mortality rates are extensively high (~40%) for device-associated Candida spp. infections and US$2.6 billion is the annual expenditure on antifungal therapies in the United States alone. Every year, about 10 million people are under threat of receiving these implant related infections when they are exposed to different medical devices such as venous catheters, urinary catheters, prosthetic heart valves and artificial joints as a result of adherent microbial colonies on the surfaces of these devices. Microbial communities typically grow on the most widely used surgically implanted devices. Furthermore, these medical apparatuses can malfunction due to the biofilm developed inside, resulting in
defective valve mechanisms. Importantly, *Candida* spp. biofilms are less susceptible to a number of antimicrobial agents and thus lead to more intricate treatment of the infected patients requiring surgical removal of the biofilms and replacement of the infected devices.\textsuperscript{157, 158}

There are limited classes of antifungal drugs that can inhibit the production of the fungi and hence there is a crucial need to develop new remedies. Remarkably, in the past two decades, researchers have only developed one new class of antifungal agents.\textsuperscript{159} The few antifungals that are available can be categorized into three main classes to *C. albicans* bloodstream infections: azoles (fluconazole, itraconazole) and polyenes (amphotericin B) and echinocandins (caspofungin). These drugs mainly target the fungal sterol ergosterol\textsuperscript{160}, thereby damaging the fungal cell wall\textsuperscript{161, 162}. Unfortunately, they exhibit poor efficacy due to drug resistance (azoles), high toxicity (amphotericin B) and rare oral availability (polyenes, echinocandins).\textsuperscript{160, 163, 164} Thus the investigation and exploration of effective treatments and new antifungal drugs need serious attention\textsuperscript{165}. The fungal PS synthase, Cho1p, represents a promising new drug target, which was revealed by the lab of our collaborator, Dr. Todd Reynolds in the University of Tennessee, Department of Microbiology. The reason for this is that, the fungal Cho1p enzyme catalyzes the conversion of cytidyldiphosphate diacylglycerol (CDP-DAG) in the presence of serine into PS as well as the byproduct cytidyldiphosphate (CMP)\textsuperscript{146, 166}. The key is that this transformation occurs in *Candida* species, but not in mammals, which instead use two PS
synthase enzymes, Pss1p and Pss2p, to exchange head groups from PC or PE, respectively, through reaction with serine to produce PS. Since mammalian and fungal systems follow two different biosynthetic routes for production of PS, an inhibitor of Cho1p could be very specific for fungi but ineffective against the mammalian PS synthases, Pss1p and Pss2p. (Figure 4.1). This also depicts that PS is produced only through the de novo pathway in the case of fungal cells whereas PE and PC can be made via both Kennedy and de novo pathways in mammals.

For this reason, we set out to develop inhibitors of the PS synthase Cho1p as a first step towards a therapeutic treatment and/or biological tool for this enzyme. Here, preliminary studies with our clickable serine analogs described in Chapter 3 suggested that modified versions of serine may effectively inhibit this enzyme. Additionally, it is noteworthy that Bahtiar et al., demonstrated O-t-Butyl-L-serine methyl ester hydrochloride (H-Ser(tBu)-OMe-HCl) as a promising as an inhibitor of osteoclastogenesis, perhaps by inhibiting sphingolipid biosynthesis. Therefore, L-serine analogs may emerge as effective PS synthase inhibitors that can be used for antifungal therapy.

4.2 Determination of PS Synthase Activity

To measure the PS synthase activity directly in vitro, our collaborator Dr. Todd Reynolds lab has developed a PS synthase assay that can be exploited PS synthase inhibition.
Figure 4.1: Different routes for production of PS in mammalian and yeast cells. Two well-known pathways used by yeast and mammalian cells to produce phospholipids. 1) The de novo pathway is different whereas the Kennedy pathway is similar for two kinds of organisms. Fungi use Cho 1p PS synthase to synthesize PS from serine (Ser) and CDP-DAG, whereas mammals use Pss1p and Pss2p to synthesize PS from serine and PC or PE respectively.

In this assay, $[^{3}\text{H}]-\text{L-serine}$ is reacted with CDP-DAG in the presence of a *Candida* membrane extract containing Cho1p (Scheme 4.1). The lipid products are then separated by thin layer chromatography (TLC), and scintillation counting is used to detect the abundance of $[^{3}\text{H}]-\text{labeled PS}$ products. To exploit this assay to evaluate inhibition, this is run in the presence (study) and absence (control) of prospective inhibitors at varying concentrations. Thus, competition with $[^{3}\text{H}]-\text{L-serine}$ with varying concentrations of serine analogs will determine their ability to inhibit the production of $[^{3}\text{H}]-\text{PS}$. Here, wild type (WT) and *cho1ΔΔ* (*C. albicans* with Cho1 knocked out) strains are employed as positives control for complete inhibition of PS synthesis. After incubation at 37 °C for a specific time (5-10 min), the unincorporated $[^{3}\text{H}]-\text{L-serine}$ is washed away and only the chloroform soluble lipid product, PS is collected. After drying overnight, PS with incorporated $[^{3}\text{H}]-\text{L-serine}$ is counted via a scintillation counter to determine enzyme activity. Compounds that decrease the production of $[^{3}\text{H}]-\text{PS}$ will be further tested by using them as competitors for deuterium labeled serine in membranes.

4.3 Synthesis of L-Serine Analogs

We designed L-serine analogs (Scheme 4.2) with modifications at either the amine or hydroxyl groups of serine. For modification of the hydroxyl group, this group was converted into an ether when attaching new functionality. For alteration of the amine group, we studied the clickable serine analogs described in and added other groups to the nitrogen through alkylation as well. Our
synthesis started with L-serine methyl ester hydrochloride (4.6) which was treated with boc anhydride in the presence of triethylamine to protect the amino group (Scheme 4.3). Next, the hydroxyl methylation was carried out using methyl iodide and sodium hydride. Finally, the boc group and the methyl ester group were deprotected using TFA in DCM to obtain compound 4.1. To synthesize compounds 4.2, 4.3 and 4.4, a general protocol was followed. In the first step, compound 3.3 was treated with alkyl halide in presence of potassium carbonate in DMF to obtain the desired intermediate (Scheme 4.4). These were then deprotected using either hydrochloric acid in dioxane-DCM or TFA-DCM mixture. The reason for switching from TFA to HCl is discussed in the next section.

Figure 4.2: L-serine analogs synthesized for PS synthase assay.
Scheme 4.3: Synthesis of serine analogs with modification of hydroxyl group.

Scheme 4.4: Synthesis of serine analogs with modification of amino group.

4.4 PS Synthase Assay Results

A number of compounds have been synthesized by our lab of which a few were already described labeling of phospholipids in the previous chapter. Many of these analogs have been assessed for PS inhibition by Chelsi Danielle Cassilly in Dr. Todd Reynolds lab in the Microbiology Department of the University of Tennessee. The compound we first studied for PS synthase inhibition was 4.1, which is an O-methoxy serine analog, and it showed very low inhibition activity (Figure 4.3). At 25 mM concentration, the analog 4.1 only reduced the activity by ~20%.
Figure 4.3: L-serine analogs assessed through a PS synthase assay demonstrating decreased $[^3]H$-PS s in the presence of an effective inhibitor.
Next, compound 3.1 showed a promising indication as an inhibitor when 25 mM of this compound was treated with protein in the presence of natural \(^3\text{H}\) labeled serine. While this concentration is too high for this analog to act as a suitable drug candidate for *candida* associated diseases, it does provide some activity that could be optimized through further probe modification. Similar results were observed for compound 4.2, which is a product of mono-methylation of serine at the amine. This exhibited pronounced inhibition activity at 25 mM as well. We also wanted to see the effect of alkylation degree on inhibition of PS synthase assay. For this purpose, we synthesized the trialkyl substituted serine analog 4.3, and this compound showed similar results to 4.3 at 25 mM. Interestingly, at 5 mM it showed a slight decrease (less than 10%) in PS production.

After obtaining reduced production of radiolabeled PS with these N-alkylated serine analogs, we decided to explore additional compounds in which the amine position was modified. Next, N-benzyl serine analog 4.4 was evaluated through the PS synthase assay, which exhibited significant inhibition of PS synthase at 25 mM concentration (Figure 4.3A). The PS production was reduced by almost 90% of the original value of PS produced in untreated sample. Moreover, for the first time, we noticed that 5 mM of 4.4 showed a significant decrease in PS production, leading to an almost 50 % decrease in PS production. However, when the same assay was repeated (Figure 4.3B), inhibition was not as significant since 5 mM of compound 4.4 reduced PS production by only, which
was unexpected since the first assay provided satisfactory results. We have remade and retested this compound multiple times and the results have been variable. One reason for this might be the trifluoroacetate salt of serine we employed in the assay, which could cause problems such as protein precipitation and may thus yield misleading results. Next, we attempted compound 4.5 containing an azido-propyl chain attached to the amine. Figure 4.4 shows that the PS assay was run three times with this compound and every time we observed pronounced inhibition of PS synthase at 5 mM concentration. To test the counter-ion effect of the salt of compound 4.5, we used hydrochloric acid in place of trifluoroacetic acid in the final deprotection to synthesize 3.2 and evaluated this compound using the PS synthase assay. The reason behind changing the counter-ion is that trifluoroacetate can cause precipitation of proteins resulting in nonfunctional protein in solution leading to ambiguous results, whereas chloride ion does not have such an effect on proteins. Figure 4.4A depicts that the first trial yielded significant inhibition of PS synthase at only 500 micromolar concentration of 3.2, which was the best result thus far (~60% reduced activity). Unexpectedly, we didn’t see the same inhibition activity when we ran a second trial with a new batch of 3.2 (Figure 4.4B).
Figure 4.4: PS synthase assay results with compounds 3.2 and 4.5.
4.5 Conclusion

The investigation for active compounds towards PS synthase inhibition is still in progress. So far, we have synthesized a number of serine analogs and showed the simple modification at different positions of the amino acid may lead to inhibition. Our group is working on making cysteine analogs, which recently showed better results than serine analogs for PS synthase inhibition. Recent, PS synthase assay result showed that our cysteine analogs compete with natural cysteine in PS assay. Tanei Ricks from our lab currently is synthesizing a number of cysteine analogs at the amine and thiol groups to show better inhibition of PS synthase.

4.6 Experimental

Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. L-serine methyl ester hydrochloride was purchased from Fisher Scientific. CDP-DAG and $^3$H serine were obtained from Avanti Polar Lipids and American Radiolabeled Chemicals respectively. SiliaPrep C18 (17%, 2g, 6mL, 40-63um) was obtained from SiliCycle Inc. Dry solvents were obtained from a Pure solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian Mercury 500 and 600 spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF mass spectrometer with high-resolution capabilities.
The scintillation counter is a Packard TriCarb 2900TR Liquid Scintillation Counter used for quantification of radio labeled PS.

(\textit{S})-methyl 2-((\textit{tert}-butoxycarbonyl)amino)-3-hydroxypropanoate (4.7). To a 100 mL round bottomed flask, was added L-serine methylester hydrochloride (4.6) (0.120 g, 0.771 mmol), trimethylamine (\textit{Et}_3\text{N}) (0.172 g, 1.7 mmol) and boc anhydride (0.168 g, 0.771 mmol) in 20 mL dry tetrahydrofuran (THF). The reaction mixture was allowed to stir for overnight. After rotary evaporation of THF, 50 mL of water was added and the aqueous layer was extracted with DCM (3 x 25 mL). The organic layers were then combined and dried with magnesium sulfate, filtered and concentrated by rotary evaporation. The compound 4.7 was passed on to next step without purification.
(S)-methyl 2-((tert-butoxycarbonyl)amino)-3-methoxypropanoate  (4.8).

Compound 4.7 (0.100 g, 0.369 mmol) was transferred to a 50 mL round bottomed flask in 10 mL of dry DMF and sodium hydride (0.035 g, 1.476 mmol) was added at 0 °C under a nitrogen atmosphere. After 30 min of stirring, methyl iodide (0.525 g, 1.6 mmol) was added to the reaction mixture and the reaction was stirred overnight. Next, 25 mL of water was added and the aqueous solution was extracted with DCM (3 x 25 mL). The organic layers were then combined and dried with magnesium sulfate, filtered and concentrated by rotary evaporation. The compound was passed on to next step without purification.

( S)-1-carboxy-2-methoxyethanammonium  2,2,2-trifluoroacetate  (4.1).

Compound 4.8 (25.0 mg, 0.108 mmol) was dissolved in DCM (1.5 mL) and treated with trifluoroacetic acid (5.0 mL), and the mixture was allowed to stir for 8h. After that, the solvent was removed by rotary evaporation, and a reverse phase C18 column with 5% methanol in water provided 4.1 (10.15 mg, 81% yield) as a white solid. $^1$H NMR (500 MHz, CD$_3$OD): δ 3.89 (m, 1H), 3.81 (t, $J$ = 4.1 Hz, 1H), 3.36 (m, 1H), 2.75 (s, 3H). HRMS-DART [M+H]$^-$ calcd for C$_4$H$_{10}$NO$_3$, 120.0655; found 120.0684.
**tert-butyl O-(tert-butyl)-N-methyl-L-serinate (4.9).** *O-tert-Butyl-L-serine tert-butyl ester hydrochloride (3.3, 0.200 g, 0.788 mmol)* was washed with 5% ammonia solution and extracted with DCM (3 x 25 mL). The DCM layers were combined in a 250 mL RBF, the solvent was removed by rotary evaporation, and the contents were placed under high vacuum. Next, 200 mL of dry DMF was added, followed by anhydrous potassium carbonate (0.239 g, 1.734 mmol), and the mixture was allowed to stir for 30 min. Next, iodomethane (0.134 g, 0.935 mmol) was added. The reaction was then allowed to stir overnight, after which ice-cold water (200 mL) was added, and the aqueous portion was extracted with DCM (3 x 25 mL). The organic layers were then combined and dried with magnesium sulfate, filtered and concentrated by rotary evaporation. The compound was passed on to next step without any purification.

The same reaction procedure was followed for making compound 4.10 and 4.11. For synthesizing 4.10, iodomethane (0.415 g, 2.81 mmol) was treated with 0.200 g (0.788 mmol) of 3.3 whereas 0.178 g (0.788 mmol) benzyl bromide was reacted with 0.200 g (0.788 mmol) of 3.3 to obtain 4.11.
(S)-1-carboxy-2-hydroxy- \( N \)-methylethanammonium 2,2,2-trifluoroacetate (4.2). Compound 4.9 (25.0 mg, 0.108 mmol) was dissolved in DCM (1.5 mL) and treated with trifluoroacetic acid (5.0 mL), and the mixture was allowed to stir for 8h. After that, the solvent was removed by rotary evaporation, and a reverse phase C18 column with 5% methanol in water provided 4.2 (22.40 mg, 89% yield) as a light yellowish liquid. \(^1\)H NMR (500 MHz, CD\(_3\)OD): \( \delta \) 4.20 (m, 1H), 3.78 (m, 2H), 3.55 (s, 3H). HRMS-DART [M+H] - calcd for C\(_4\)H\(_{10}\)NO\(_3\)\(^+\), 120.0655; found 120.1002.

(S)-1-carboxy-2-hydroxy-\(N,N,N\)-trimethylethanammonium 2,2,2-trifluoroacetate (4.3). To a solution of 4.10 (0.025 g, 0.096 mmol) in 1.5 mL DCM, added 5.0 mL of trifluoroacetic acid and stirred for couple of hours. A white product for 4.3 was obtained after reverse phase column with 10% methanol in
water employed 19.53 mg (92% yield). $^1$H NMR (300 MHz, D$_2$O): £ 4.06 ((d, $J$ = 4.4 Hz, 3H)), 3.93 (t, $J$ = 4.6 Hz, 1H), 3.14 (s, 9H). HRMS-DART [M+H]$^-$ calcd for C$_6$H$_{14}$NO$_3^+$, 148.0973; found 147.1124.

(!S)-N-benzyl-1-carboxy-2-hydroxyethanammonium 2,2,2-trifluoroacetate (4.4). To a solution of 4.11 (0.025 g, 0.0813 mmol) in 1.5 mL DCM, was added 5.0 mL of trifluoroacetic acid and the reaction stirred for 9h. A reverse phase column with 10% methanol in water employed compound 4.4 as colorless solid (23.88 mg, 95% yield). $^1$H NMR (300 MHz, D$_2$O): £ 7.52-7.45 (m, 5H)), 4.30 (s, 2H), 4.02 (m, 2H), 3.88 (m, 2H). $^{13}$C NMR (126 MHz, CD$_3$OD): £ 170.76, 154.89, 130.42, 129.94, 129.67, 129.20, 62.05, 60.85, 59.02, 49.94. HRMS-DART [M+H]$^-$ calcd for C$_{10}$H$_{14}$NO$_3^+$, 196.097; found 196.097.
(S)-3-azido-\textit{N}(1-carboxy-2-hydroxyethyl)propan-1-ammonium chloride (4.5). Protected azido-serine 3.5 (25.0 mg, 0.083 mmol) was dissolved in DCM (1.5 mL) and treated with trifluoroacetic acid (3.5 mL, 8 M), and the mixture was allowed to stir for 8h. After that, the solvent was removed by rotary evaporation, and a reverse phase C18 column with 10% methanol in water provided 4.5. All the peaks for 4.5 matched with that of compound 3.2.

**Phosphatidylserine Synthase Assay**

This procedure was done as described in literature\textsuperscript{172} with minor alterations. Wild type \textit{cho1ΔΔ} \textit{candida albicans} cultures were grown over night and then diluted into 1 L YPD, approximately 0.1 OD\textsubscript{600}/mL. These cultures were shaken at 30 °C for 6 to 10h. Cells were then harvested by centrifugation at 6,000 x g for 20 minutes. Pellets were then transferred to 50 mL conical tubes and washed with water and re-pelleted. Supernatant was removed and the wet weight of the samples was taken. Cell pellets were stored overnight in -80 °C. The following day, a cold mixture of 0.1 M Tris-Cl pH 7.5, 5 mM β-mercaptoethanol (BME), 10% glycerol, and protease inhibitors (phenylmethylsuphonylfluoride (PMSF), leupeptin, and pepstatin) was added to the frozen pellets (1mL/g [wet weight]) and allowed to thaw on ice. Cells were lysed using a French Press (three passes at approximately 13,000 lb/in\textsuperscript{2}). The homogenate was centrifuged at 4 °C for 5 minutes at 3,000 rpm to clear unbroken cells and heavy material. Supernatant was then spun again at 27,000 g for 10 minutes at 4 °C. For some experiments, the resulting supernatant was then spun at 100,000 g to collect the lower density
membranes. Pellets were resuspended in 500 μL to 1 mL of 0.1 M Tris-Cl pH 7.5, 5 mM β-mercaptoethanol (BME), 10% glycerol, and protease inhibitors. This mixture was aliquoted into microcentrifuge tubes and homogenized to break apart clumps, keeping on ice as much as possible. Total crude protein concentration was determined using a Bradford Assay. The optimal assay mixture contained 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 0.5 mM MnCl₂, 0.1 mM CDP-DAG (Avanti Polar Lipids, Alabaster, AL) added as a suspension in 1% to 20% Triton X-100, and 0.4 to 0.5 mg protein in a total volume of 0.1 mL. The PS synthase assay was performed by monitoring the incorporation of 0.5 mM l-serine spiked with 5% by volume [³H]-l-serine (~20 Ci/mmol) into the chloroform-soluble product at 37 °C for a predetermined amount of time. The reaction was terminated by the addition of 1 mL chloroform: methanol (2:1). Following a low-speed spin, 800 to 1000 μL of the supernatant was removed to a fresh tube and washed with 200 μL 0.9% NaCl. Following a second low-speed spin, 400 to 500 μL of the chloroform phase was removed to a new tube and washed with 500 μL of chloroform: methanol: 0.9% NaCl (3:48:47). Following a third low-speed spin, 200 to 300 μL was transferred into scintillation vials (Thermo Fisher Scientific, San Jose, CA). Tubes were left open in the hood until dried fully. The next day, 2.5 mL scintillation fluid was added to each tube and run through the scintillation counter.
4.6.1 List of Spectra

Spectra 4.1: $^1$H NMR and mass spectra of compound 4.1. (S)-1-carboxy-2-methoxyethanammonium 2,2,2-trifluoroacetate.
Spectra 4.2: $^1$H NMR and mass spectra of compound 4.2. (S)-1-carboxy-2-hydroxy- N-methylethanammonium 2,2,2-trifluoroacetate (4.2).
Spectra 4.3: $^1$H NMR and mass spectra of compound 4.3. (S)-1-carboxy-2-hydroxy-$N,N,N$-trimethylethanammonium 2,2,2-trifluoroacetate (4.3).
Spectra 4.4: $^1$H and $^{13}$C NMR of compound 4.4. (S)-N-benzyl-1-carboxy-2-hydroxyethanammonium 2,2,2-trifluoroacetate (4.4).
Spectrum 4.5: Mass spectra of compound 4.4. (S)-N-benzyl-1-carboxy-2-hydroxyethanammonium 2,2,2-trifluoroacetate (4.4).
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VITA

Shahrina Alam was born in Dhaka, Bangladesh. After finishing her high school education, she first chose physics as her major at the University of Dhaka, Bangladesh but later she realized that chemistry is fascinating and more attractive since any physical or chemical change can be visualized as well as monitored using different techniques. After finishing her B.Sc. (Hons) degree, she planned to do higher studies and came to Tennessee State University to pursue her Master's degree. Here, she worked on "Boron Chemistry", under Dr. Mohammad Almasum. Her research topic was on microwave-enhanced cross-coupling of allyltrifluoroborates and aryl halides to produce trans-\(\beta\)-methyl styrenes and she was able to publish a paper in Tetrahedron Letters. She received her Master's degree in Organic Chemistry in 2009 and in the same year she returned her home country, accepted an offer for Lecturer position at American Int'l University-Bangladesh. While serving as a teacher, she realized her desire to pursue higher studies in chemistry and came back to the USA to pursue the Ph.D. degree at the University of Tennessee. After joining the department, she was determined to join Dr. Best’s research lab, mainly because of his research topic and personality. During her Ph.D. career, she worked as a graduate teaching assistant, published few papers in peer-reviewed journals and participated in the national chemical conference.