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UNIVERSITY OF CALIFORNIA SAN DIEGO

Cellular Detection of Oxidized Lipids Guided by Insights from Membrane Mimetic
Chemistry

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy
in

Biochemistry and Molecular Biophysics

by

Jacob Aaron Vance

Committee in charge:

Professor Neal Devaraj, Chair

Professor Itay Budin

Professor Michael Burkart

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Professor Jin Zhang

2025

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University of California San Diego

2025

Dedication

I dedicate this dissertation to Renee Niles. In a more just world, you would be defending your dissertation this year, too.

Epigraph

“This is rather as if you imagine a puddle waking up one morning and thinking, *‘This is an interesting world I find myself in — an interesting hole I find myself in — fits me rather neatly, doesn’t it? In fact, it fits me staggeringly well, must have been made to have me in it!’* This is such a powerful idea that as the sun rises in the sky and the air heats up and as, gradually, the puddle gets smaller and smaller, it’s still frantically hanging on to the notion that everything’s going to be all right, because this world was meant to have him in it, was built to have him in it; so the moment he disappears catches him rather by surprise. I think this may be something we need to be on the watch out for.”

—Douglas Adams

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Notes on Chapters

Chapter two, in full, is a reprint (with co-author permissions) of the material as it appears in the publication J. A. Vance, N.K. Devaraj “Membrane Mimetic Chemistry in Artificial Cells,” *J. Am. Chem. Soc.* 2021, 143, 22, 8223–8231. The dissertation author was the primary researcher and author of this material.

Chapter three, in full, is a reprint (with co-author permissions) of the material as it appears in the publication J. A. Vance, W. A. Chen, P. Nepachalovich, C. Knittel, M. Fedorova, N. K. Devaraj, *Angew. Chem. Int. Ed.* 2025, e202512578. <https://doi.org/10.1002/anie.202512578>. The dissertation author was the primary researcher and author of this material.

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Abstract of the Dissertation

Cellular Detection of Oxidized Lipids Guided by Insights from Membrane Mimetic Chemistry

by

Jacob Aaron Vance

Doctor of Philosophy in Biochemistry and Molecular Biophysics

University of California San Diego, 2025

Professor Neal Devaraj, Chair

Lipid membranes are the boundaries of life, concentrating information and energy to sustain entropy-reducing processes. Here, I discuss membrane mimetic chemistry as a framework for designing dynamic compartments to host artificial cells. Coupling reactions such as the hydroxylamine/carbonyl (oxime) ligation allow rapid generation and growth of artificial membranes from soluble precursors with unexpectedly rapid kinetics. The robustness of these coupling reactions motivates the development of chemical tools for probing lipid carbonyls in cells. I discuss the design of probes for detecting the presence of truncated, aldehyde-containing phospholipids in living cells. We show that oxime ligation to oxidized lipids, OxiLox, provides a

chemically precise readout of phospholipid aldehyde burden in cells undergoing ferroptosis.

1 Introduction

1.1 Purpose and scope

This dissertation presents a method for detection, imaging, and quantification of oxidatively damaged phospholipids in living cells. Phospholipid aldehydes result from the oxidative truncation of unsaturated acyl chains, existing as very low-abundance species embedded in the membranes of cells.^[1] These electrophilic molecules have been implicated in various human diseases, and their precise activities within cells are an area of active research.^[2–4] Inspired by insights gleaned from studies in membrane mimetic chemistry (chapter 2), we designed a method for detecting these species in living cells by ligating them to fluorescent probes by oxime chemistry. Our method anchors the fluorophore at the site of membrane damage for confocal imaging and forms a stable oxime derivative that can be identified by LC-HRMS.

1.2 Background and probe design rationale

Membrane mimetic chemistry is a methodological stance and set of design principles with the goal of the construction of cell-sized, dynamic compartments that can host complex reaction networks and that undergo generational population-scale incremental changes under selective pressure. In our 2021 perspective defining membrane mimetic chemistry, which is reprinted herein as Chapter 2, we emphasized that a key step on the path to artificial cell membranes is design of chemical reactions for generating and growing lipid membranes from precursors that, by themselves, do not form membranes in water (Figure 2.1). The Devaraj lab has shown that several coupling reactions can be adapted for the design of systems for de novo synthesis of dynamic membrane compartments.^[5,6]

An important insight from these studies is that phospholipid membranes can concentrate and template the synthesis of additional monomers, accelerating reaction rates. In 2022, Flores et al. showed that polar head groups with truncated acyl chains terminating in hydroxylamines react in water with short-chain alkyl aldehydes, spontaneously forming cell-sized vesicles in water composed of unnatural oxime derivatives of phospholipids.^[7] The rapid speed of the reaction (completing in 30 minutes) was a surprising feature, given that nucleophile-carbonyl condensation reactions typically exhibit sluggish kinetics at neutral pH in the absence of a catalyst.^[8] For this reason, in-cell applications of the oxime ligation for bioconjugation have been limited. In light of the findings from Flores et al., we hypothesized that the oxime ligation may similarly function to rapidly label membrane-embedded aldehydes that result from lipid peroxidation, providing a direct chemical readout.

1.3 Principal findings

In chapter 3, I reprint our publication, “Targeted Detection of Phospholipid Aldehydes in Living Cells by Oxime Ligation.” In this work, we describe the design, synthesis, and testing of tetramethylrhodamine (TAMRA) probes bearing hydroxylamine groups on a variety of linkers (Figure 3.3). These probes rapidly conjugate to phospholipid aldehydes in silica-supported lipid bilayers (SSLBs) and in living cell membranes (Figure 3.5, Figure 3.9). With properly designed probes, oxime ligation proceeds efficiently in intact membranes, and unreacted dye is washed out more readily than the membrane-anchored oxime adducts. The oxime derivatives are also stable during extraction and visible by mass spectrometry, allowing LC/MS quantification and direct detection of the same lipid species observed in imaging experiments (Figure

3.10). Importantly, our LC-MS analysis showed that, in line with previous work, ferroptosis results in the enrichment of truncated plasmenyl phosphatidylethanolamines.^[9] In both model membranes and living cells, probe retention, measured by fluorescence intensity, increases in step with the accumulation of derivatized lipid aldehydes. Taken together, these experiments show that phospholipid aldehydes persist in a steady state, with a fraction remaining free and available for reaction with nucleophiles rather than being fully metabolized or sequestered as protein adducts. Our work also shows that the pool of lipid aldehydes increases significantly under oxidative stress induced by the ferroptosis inducer RSL3 (Figure 3.9). This balance between free and neutralized aldehydes has important consequences, as enzymatic neutralization directly influences cell survival (Figure 3.11).

Together, these results highlight the importance of considering the unique chemical environment of cellular membranes in the design of bio-orthogonal reactions that aim to label membrane lipids. Hydrophobic probes with longer alkyl linkers show enhanced reactivity toward aldehyde lipids, but this advantage comes with a cost: increased nonspecific association that can obscure the signal from derivatized products. Insights of this type—arising from the interplay between probe design, membrane chemistry, and cellular metabolism—illustrate how principles drawn from membrane mimetic chemistry can be leveraged to create effective tools for interrogating lipid activities in living cells.

2 Membrane Mimetic Chemistry in Artificial Cells

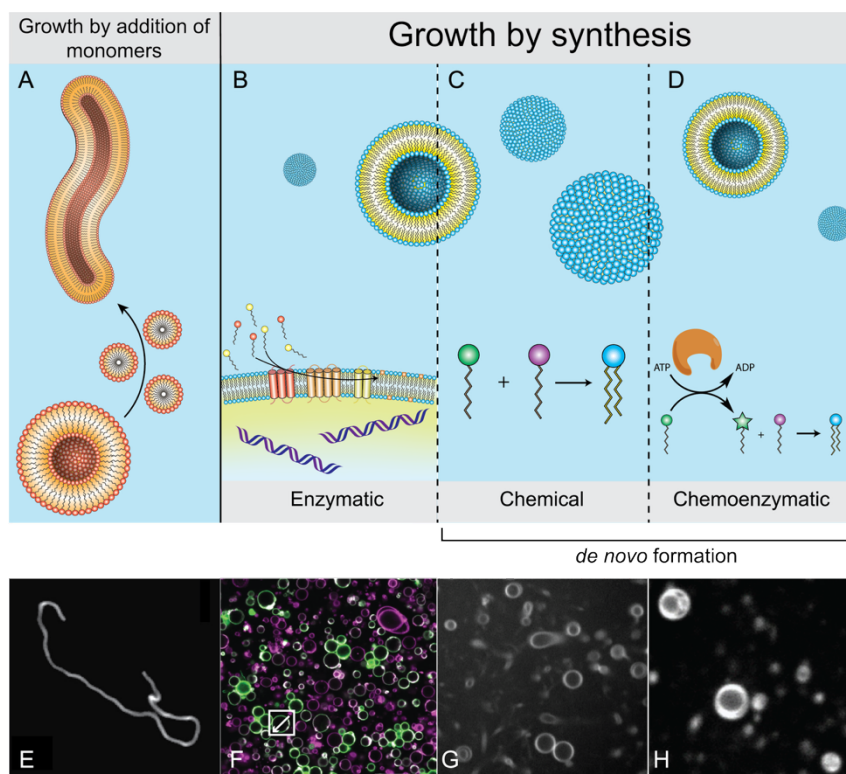


Figure 2.1 Growth in artificial cell membranes.

(A–D) Schematic representations of methods for growth in artificial cell membranes: (A) Growth can be induced in fatty acid vesicles by introducing fatty acid micelles to the extravesicular environment. (B) Synthesis of new phospholipids through reconstitution of membrane-bound enzymes. (C) Formation and growth of membranes by in situ chemical synthesis of phospholipids. (D) Formation and growth of membranes through enzymatic activation of lipid precursors followed by chemical coupling. (E–H) Images of membrane expansion by the methods represented in panels A–D: (E) Growth and tubulation of a fatty acid vesicle containing RNA upon addition of 5 equiv of oleate micelles. [Adapted from ref 24. Copyright 2009 American Chemical Society.] (F) Enzymatic production of membrane-forming phospholipids. Vesicles encapsulate a cell-free expression system and a plasmid encoding phospholipid synthesis enzymes. DOPS is then produced enzymatically when vesicles are supplied with oleoyl-CoA. The phosphatidylserine-binding protein LactC2-eGFP binds to vesicles that successfully produced DOPS, staining such membranes in green. [Adapted with permission from ref 31. Copyright 2020, licensed under CC BY 4.0.] (G) Formation of phospholipid membranes by nonenzymatic synthesis. Esterification of lysophospholipids by synthetic acyl thioesters is carried out in water, leading to in situ self-assembly of natural phospholipid vesicles as described in ref 38. (H) Formation of vesicles by chemoenzymatic synthesis. The soluble enzyme FadD10 is used to form fatty acyl adenylates, which react with amine-functionalized lysolipids to yield membrane-forming phospholipids that spontaneously self-assemble in water to form lamellar vesicles. [Adapted with permission from ref 39. Copyright 2020, licensed under CC BY 4.0.]

2.1 Introduction

The development of artificial cells is a principal task of “bottom-up” synthetic biology, which aims to build biomimetic systems using non-living precursors.^[10] The

effort to reproduce life-like characteristics synthetically will lead to an improved understanding of biological processes and the development of next-generation tools in biotechnology and pharmaceuticals.^[11] As living cells are extraordinarily complex, the term artificial cell has generally been applied to systems that mimic a small subset of the characteristics of living cells. Consequently, systems that use this term can vary quite significantly in their properties and may not obviously resemble living organisms. While advances in recombinant transcription/translation systems have led to a surge of interest in cell-free synthetic biology,^[12–14] biomimetic systems that lack defined compartments have significant disadvantages. Compartmentalization, or physical separation between and within cells, is an attribute of all known organisms. Compartmentalization appears to serve many purposes in living cells, and its ubiquity suggests that it is of critical importance to life. Crucially, compartmentalization is essential for self-replicating systems to maintain or increase their complexity at the expense of free energy.^[15] We observe that, among other things, cellular compartments separate incompatible chemical reactions, house and protect genetic information, increase reaction rates by concentrating substrates and enzymes, and maintain electrochemical gradients for metabolic processes.^[16] These functions are key to cellular energy homeostasis, growth, and proliferation. Therefore, systems that attempt to replicate these fundamental biological processes in artificial cells will require the development of synthetic methods for compartmentalization.^[17]

Researchers attempting to develop artificial cells will undoubtedly look to biology for inspiration in the effort to reproduce the characteristics of life. While membraneless and protein-exclusive microcompartments have been observed in many organisms, all

life forms are defined by compartments consisting of phospholipid bilayer membranes.^[16,18,19] Because living cells rely disproportionately on phospholipid membranes for compartmentalization, synthetic biologists have frequently used lipid-based vesicles in synthetic cells.^[20] Typically, the lipid vesicles are used as stable compartments that ensure encapsulated material does not disperse, promoting colocalization of reactants. Cell membranes, conversely, are different than simple reconstituted lipid vesicles in significant ways. For instance, they are dynamic, constantly changing in chemical composition. The development of lipid membranes that more closely resemble living membranes is therefore critical to research in bottom-up synthetic biology and the development of artificial cells. Because the task of constructing life-like membranes requires the formation of self-assembling molecules from chemical building blocks, we believe there exists an opportunity to utilize biomimetic chemistry to create artificial membranes that more faithfully mimic the structure and function of living membranes.

Chemistry involving biomimetic membranes is not a new endeavor. The term membrane mimetic chemistry was popularized by Janos Fendler in the 1980s.^[21] Membrane mimetic chemistry, as described by Fendler, is the effort to utilize lipid nanostructures (e.g., membranes, micelles, vesicles, etc.) to facilitate chemical reactions. Membranes were used to compartmentalize chemistry and template reactions through self-assembly and reagent colocalization.^[22] While parallels were drawn to membrane biology (for instance, the role of membranes in photosynthesis),^[23] mimicking biological phenomena (division, growth, transport) was not the explicit objective. Conversely, chemists interested in building an artificial cell are attempting to

construct lipid-based compartments that faithfully emulate the properties of living membranes.^[24] Given the modern understanding of biological membranes and synthetic chemistry, we suggest a revival of membrane mimetic chemistry with an emphasis on the development of functional artificial cell membranes with lifelike properties.

Approaches using minimal chemical and/or biochemical elements to mimic cell membranes can be considered. Pursuing membrane mimetic chemistry will greatly advance our understanding of fundamental lipid chemistry, improve our ability to generate biomimetic membranes, and accelerate applications using lipid-based tools.^[11]

We believe that membrane mimetic chemistry is an important cornerstone of artificial cell research. While the development of an artificial cell may require reconstituting existing biological macromolecules in liposomes, we hope to emphasize that an artificial cell membrane in principle does not need to contain any material derived from living organisms. Drawing inspiration from origin-of-life research, we suggest that it may be possible to mimic many existing cellular processes using minimal chemical systems that do not obviously resemble their living counterparts.^[24] Here we discuss recent advances in membrane mimetic chemistry with respect to properties of phospholipid membranes that we view as fundamental to biomimetic compartments. We discuss methods for the generation and growth of lipid vesicles. As a key property of cells is their ability to reproduce, we then turn our attention to controlled division of artificial membranes. While phospholipid membranes act as barriers, an artificial cell will need to be able to control the exchange of material with the surrounding environment. We comment on methods for controlling membrane permeability to small molecules and ions. Finally, we examine lipid mesophases as a potential alternative to lamellar

vesicles for compartmentalization and mimicry of membrane-bound organelle function. Simultaneously, we also look to the near future in identifying unmet needs and potential opportunities, with an aim to inspire research efforts in this emerging area.

2.2 Membrane generation and growth

In the effort to generate life-like membranes in the laboratory, it is helpful to consider the origin of living cell membranes. The current dogma in biology is that all living membranes originate from pre-existing membranes.^[25] This is supported by the fact that diacyl phospholipids are generally synthesized in living organisms by membrane-bound acyltransferases. Hence, the reactions that produce membrane-forming monomers can only occur within membranes themselves. If this principle of membrane continuity holds true, it suggests that all living phospholipid membranes are descendants of an ancient lipid membrane. The circumstances that led to the genesis of this primordial membrane are obscured by billions of years of evolution, but several possibilities for its origin have been suggested. One hypothesis is that modern cell membranes arose gradually from more primitive cells that possessed membranes composed of simpler amphiphiles.^[26] This possibility has prompted researchers to consider alternative membrane-forming amphiphiles and question whether phospholipids are in fact necessary for the assembly of life-like lipid-based membranes.

Several researchers have theorized that the first biological membranes were composed primarily of fatty acids.^[27–30] The abiotic generation of fatty acids has been deemed more prebiotically plausible than the formation of diacyl phospholipids. Indeed, fatty acids readily form vesicles, can be formed in Fischer–Tropsch-like reactions, and have even been found on chondraceous meteorites.^[31,32] Additionally, fatty acid vesicles

have a key advantage over phospholipid vesicles: growth can be induced in fatty acid vesicles by simple addition of monomers (Figure 2.1 A,E). In a seminal study, the Szostak lab observed that addition of fatty acid micelles to multi-lamellar vesicles triggered vesicle growth by the formation of tubular structures.^[33] The Szostak group has also demonstrated that fatty acid vesicles can grow due to the osmotic pressure changes that take place upon encapsulation of high concentrations of negatively charged biopolymers like RNA.^[34] Such simple physical phenomena may explain the emergence of Darwinian evolution among protocells encapsulating ribozymes. Vesicles formed from fatty acids have also been shown to support some simple biochemical reactions, including ribozyme-catalyzed reactions.^[35] Facile methods for inducing growth have made fatty acid vesicles important for origin-of-life research and may make them attractive platforms for some synthetic biology applications.

Fatty acid vesicles, however, are not generally capable of harboring many of the reactions that are typically associated with existing life. They suffer from an intolerance to divalent cations, are unable to support proton gradients, and can inhibit the action of polymerases.^[24] Furthermore, fatty acids tend to have very high critical vesicle concentrations (e.g., vesicles form from decanoic acid only above 40 mM fatty acid) and show limited pH stability. Some of these issues can be alleviated by using vesicles composed of mixtures of single-chain amphiphiles. For example, it has been shown that low concentrations of long-chain fatty acids can significantly lower the concentration at which vesicles form from short-chain fatty acids.^[36] The inclusion of fatty alcohols can also stabilize fatty acid vesicles to pH changes.^[30] It remains the case, however, that because many enzymes are incompatible with high concentrations of simple

amphiphiles, fatty acid vesicles are unlikely to be adequate platforms for artificial cells that host complex enzyme-dependent reactions.

Because fatty acid vesicles are incompatible with many biochemical reactions, it has been theorized that mixed fatty acid and phospholipid membranes may have represented an intermediate stage in the evolution of modern membranes. Budin et al. have demonstrated that fatty acid vesicles containing a percentage of phospholipid will grow when vesicles containing a higher ratio of fatty acid are added to the surrounding medium.^[37] That is, hybrid phospholipid/fatty acid vesicles can accumulate additional fatty acid amphiphiles at the expense of pure fatty acid vesicles, constituting a form of lipid competition between vesicle species. The result suggests that the ability to generate dialkyl lipids would have conferred a selective advantage on protocellular membranes. By coupling recent advances in the generation of phospholipids either chemically or enzymatically, it may be possible to create vesicles that can generate phospholipids catalytically and grow at the expense of vesicles that are unable to generate phospholipids.

While origin-of-life researchers have focused their attention on membranes consisting of single-chain amphiphiles due to prebiotic plausibility, synthetic biologists tend to employ vesicles composed mainly of phospholipids. Methods for generating phospholipid-based liposomes with static membranes have been extensively discussed in a recent review by Wang et al.^[38] Growth of liposomes requires either in situ formation of monomers from non-membrane-forming precursors or membrane fusion.^[39] Here, we focus on recent membrane mimetic chemistry used for producing dynamic lipid membranes capable of growth by lipid synthesis (Figure 2.1B). A potentially

straightforward approach for creating membranes capable of growth and lipid remodeling is to reconstitute the natural cellular lipid synthesis machinery (Figure 2.1). Demonstrating this concept, Exterkate et al. recently reconstituted bacterial enzymes involved in membrane synthesis in liposomes, leading to the formation of phospholipids when vesicles were supplied with free fatty acids and glycerol-3-phosphate. In exciting work, Blanken et al. reconstituted enzymatic synthesis using vesicles encapsulating a cell-free expression system (Figure 2.1F).^[40] These researchers reconstituted seven enzymes from the *E. coli* Kennedy lipid synthesis pathway in phospholipid vesicles. Functionality was demonstrated by the formation of natural phospholipids such as phosphatidylethanolamine and phosphatidylglycerol in the liposomal membrane. This work validated that an enzymatic strategy can result in membranes capable of transcriptionally regulated phospholipid synthesis. The method described by these researchers requires that enzymes be expressed inside existing liposomes and that vesicles be supplied with acyl-CoA and glycerol-3-phosphate, which are normally produced enzymatically inside living cells. However, liposomes showed large heterogeneity in lipid production, and no visible vesicle growth could be observed due to the limited amount of phospholipid produced. The reconstitution of an entire lipid synthesis pathway inside liposomes is clearly challenging and may require cofactors that have not yet been characterized. Recent work has demonstrated that lipid synthesis can be highly regulated by accessory proteins, which if absent in cell-free systems might lower the efficiency of reaction.^[41]

One way to avoid the challenges of reconstituting lipid synthesis enzymes is to mimic phospholipid synthesis reactions non-enzymatically (Figure 2.1C). In past work,

our lab has generated vesicles composed of unnatural phospholipids using click chemistry and native chemical ligation and showed that these coupling reactions can drive membrane expansion.^[42–44] Related research has demonstrated that combining lipid chemical conjugation methods with a phase-transfer catalyst can result in dissipative self-assembly of liposomes.^[45] To form natural phospholipids, we attempted to emulate biological coupling reactions. While mimicking all the steps of Kennedy lipid synthesis pathway is daunting, our lab drew inspiration from another lipid synthesis pathway known as the Lands cycle or remodeling pathway.^[46] Specifically, a key step of the Lands cycle involves combining a single-chain lysophospholipid with a single-chain thioester derivative of a fatty acid to form a new phospholipid. Neither of the single-chain precursors form membranes, but the coupling product does. We sought to mimic the acylation coupling step non-enzymatically using chemoselective chemistry. By choosing reactions that work in water, the solvent in which lipid self-assembly takes place, we observed spontaneous formation of membrane-bound vesicles during the synthesis reaction. Furthermore, our approach generated lipid membranes *de novo* (i.e., in the absence of preexisting membranes), which, as discussed, is thought to not occur in biology. Ultimately, this method allowed the synthesis of natural, membrane-forming phospholipids under aqueous conditions in the absence of enzymes (Figure 2.1G).^[47] This result suggests that it may have been possible for phospholipid membranes to form without the need for a pre-existing membrane composed of simpler amphiphiles and could be applied to the generation of artificial cell membranes.

However, unlike enzymatic synthesis of diacyl phospholipids, purely chemical methods cannot be easily controlled by transcriptional regulation. Combining enzymatic

and nonenzymatic methods can allow high-yielding de novo formation of membranes from simple precursors while retaining transcriptional control (Figure 2.1D). For example, a chemoenzymatic approach demonstrated by Bhattacharya et al. resulted in the de novo formation of phospholipid membranes that could be linked to transcription, and vesicle growth could be observed (Figure 2.1H).^[48] Chemoenzymatic synthesis may therefore be an effective method for creating membranes capable of sustained and tunable growth.^[49] As discussed in a recent review by Liu et al., membranes composed entirely of peptides have also been proposed as platforms for artificial cells.^[50] Peptide vesicles could potentially be generated enzymatically, allowing for transcriptionally controlled membrane formation.

2.3 Membrane Fission

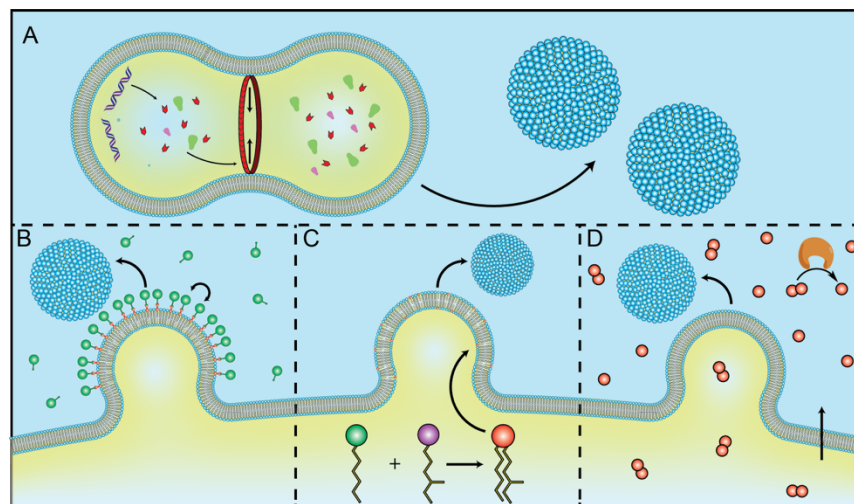


Figure 2.2 Fission of membrane-bound vesicles.

(A) Division caused by reconstitution of cellular division machinery. (B) Membrane perturbations and budding caused by recruitment of high concentrations of macromolecules to the vesicular membrane. (C) Excess membrane synthesis and membrane remodeling can lead to instabilities and membrane scission. (D) Increase in osmolarity in the extravesicular space leading to a reduction of volume encapsulated by vesicles and membrane fission.

The fission of membranes into distinct compartments is essential for many biological processes. In living organisms, membrane fission allows for cellular growth

and division and facilitates vesicular transport between and within compartments. It is highly desirable to create artificial cells capable of controlled fission because such systems could be used as models for cellular division or platforms for the in vitro reconstitution of biological phenomena such as Darwinian evolution (Figure 2.2). However, the division of membrane-bound structures requires overcoming a substantial energetic barrier to deform the membrane and eventually achieve separation.^[51,52] Fission in living cells is exceptionally complex and involves sophisticated cellular machinery such as FtsZ, actin-myosin rings, ESCRT, or dynamin.^[53] Cellular division is a highly controlled fission process that results in two compartments that are approximately equivalent in size and content. Reconstituting division machinery in its entirety in minimal synthetic cells would likely be cumbersome and impractical. Some groups have therefore tried to reconstitute only what are expected to be the most important cellular components for triggering division (Figure 2.2A). One such approach involves the in vitro reconstitution of the prokaryotic divisome. The principal components of this complex molecular machine are the contractile ring structure formed by the bacterial tubulin homolog FtsZ and several proteins, termed Min proteins, which facilitate its polymerization at the equator of the cell.^[54,55] Schwille and co-workers first reported the in vitro reconstitution of Min proteins on planar lipid bilayers, on which they observed surface wave patterns that are reminiscent of oscillations that have been observed in live bacteria.^[56] More recently, Litschel et al. and Godino et al. successfully reconstituted Min proteins in lipid vesicles (Figure 2.3A-B). They observed similar oscillatory patterns as well as membrane deformation, suggesting that reconstitution of minimal division machinery can lead to significant membrane perturbations.^[57,58] Likely

because these proteins work in concert with other proteins, many of which remain uncharacterized, there has thus far been limited success in achieving controllable fission in artificial cell membranes through these methods. Other research, however, has been spent pursuing potential methods for inducing fission in artificial cells by reconstituting processes unrelated to the cell cycle. For example, it was recently demonstrated that proteins involved in vesicular transport could induce fission in liposomes.^[59] These simpler systems may provide alternative sources of inspiration for inducing fission in artificial cellular membranes

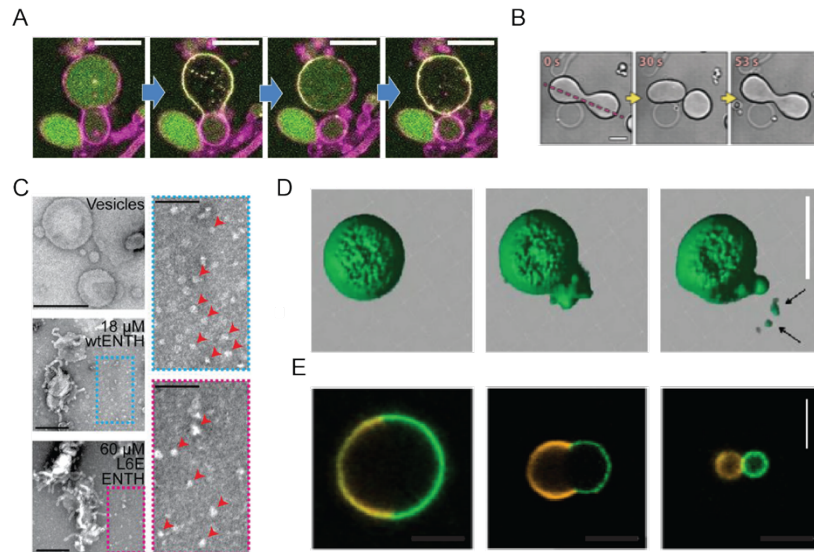


Figure 2.3 Examples of division in artificial cells.

(A-B) Bacterial Min proteins lead to significant membrane deformation when they are expressed inside vesicles. [Adapted with permission from ref 48. Copyright 2020. licensed under CC BY 4.0.] (C) Membrane tubulation and budding as a result of binding of the clathrin adaptor epsin1 N-terminal homology (ENTH) domain. Binding of ENTH to membranes is thought to increase membrane spontaneous curvature through two possible mechanisms: insertion of an amphipathic helix that causes a reduction in leaflet surface area, or steric crowding at the membrane surface that forces curvature. [Adapted with permission from ref 60.] (D) Fission by excess membrane synthesis. [Adapted with permission from ref 35.] (E) Division of phase-separated vesicles by external modulation of osmotic pressure. The liquid disordered phase is labeled with LissRhod PE (orange, $\lambda_{ex} = 561$ nm) and the liquid ordered phase with 6-FAM-labeled cholesterol-tagged DNA (green, $\lambda_{ex} = 488$ nm). Vesicles undergo fission when the osmolality of the extravesicular environment is increased through a simple enzymatic reaction. [Adapted with permission from ref 52. Copyright 2020, licensed under CC BY 4.0.]

To achieve fission of artificial cells, researchers may need to look beyond reconstituting existing machinery from living organisms. Although all known living

membranes achieve fission using complex protein-based machines, division in primitive cells likely occurred through simpler mechanisms. Understanding these mechanisms is key to understanding the evolution of life and may also be crucial to inducing fission in minimal cells. Some have suggested that division of early cells could have been driven by mechanical perturbations. Deshpande et al. demonstrated that fission could be induced through microfluidic methods that flow liposomes against sharp edge.^[60] Recent work has also shown that osmotic pressure, generated by manipulating the external solution bathing lipid vesicles, can be used to trigger fission in phase-separated giant unilamellar vesicles (Figure 2.3E).^[61] While these techniques allow for considerable control over the volume and shape of resulting daughter vesicles, primitive cell membranes capable of autonomous division without external intervention must have arisen early in the evolution of modern cells.^[62]

One clue as to how primitive cells may have divided comes from the observations of Errington and co-workers who have shown that a primitive kind of membrane division, which does not result in two equivalent daughter cells, can occur in bacteria that lack a cell wall (L-form bacteria).^[63] Such bacteria do not divide using the conventional FtsZ-reliant bacterial division machinery, so they must acquire genetic mutations that allow division while in this state.^[64] They showed that proliferation in L-form *B. subtilis* can occur after the bacterium acquires one or more mutations that cause an increase in the abundance of phospholipids, leading to excess membrane synthesis. Previous work showed that membrane scission during proliferation is dependent on the production of branched-chain fatty acids, which increase the fluidity of the resulting membranes.^[65] These results suggest a major role for lipid expansion and

composition in promoting membrane morphological changes (Figure 2.2C). This is also supported by observations that phase separated vesicles tend to undergo stimuli dependent division more readily.^[61] Logically, changes in the physical properties of membranes produced by changing phospholipid composition would likely help cells overcome the enormous energy barrier required to divide. Even complex multi-cellular organisms include remodeling of the lipid membrane as a crucial part of the process of cytokinesis.^[66] Work from our lab and others has shown that membrane expansion can drive vesicle budding at low rates, suggesting that instabilities caused by expanding the membrane surface area while maintaining vesicle volume could potentially be exploited for inducing membrane division (Figure 2.3D).^[42] Finely tuned remodeling of vesicle membranes is expected to be necessary for division of artificial cells and could result in programmed division events. More work will be needed in this area to fully understand the role that membrane composition plays in division and to explore methods for adjusting lipid content of synthetic membranes in situ. One possible approach would be to harness chemical mechanisms to remodel artificial cell membranes, either reversibly or driven through the use of chemical fuels and dissipative selfassembly.^[45,67,68]

While careful attention to the lipid membrane composition of artificial cells is likely to be necessary for controlled fission, simple interactions between membranes and macromolecules may also drive membrane deformation (Figure 2.3B).^[52] It has been shown that protein crowding at the membrane can impact cell membrane curvature and cause membrane budding. For example, work by Stachowiak and co-workers demonstrated that high concentrations of recombinant clathrin adaptor epsin1 N-terminal homology (ENTH) domain caused division of liposomes.^[69] This protein domain

was previously understood to aid in fission through shallow insertion of a hydrophobic helix into the membrane, impacting leaflet surface area. However, they showed that binding of ENTH causes membrane deformation even when the amphipathic helix responsible for membrane insertion is mutated (L6E) to impact hydrophobicity, suggesting that deformation can result from high densities of ENTH at the membrane (Figure 2.3C). They then demonstrated that fission can be driven by recruitment of high concentrations of GFP to the membrane, meaning that macromolecular crowding, regardless of protein identity, may provide a generalized mechanism for inducing fission in artificial cell membranes.^[52] More work is needed to explore how these effects could be finely tuned to produce division that is controllable and that leads to daughter cells that are similar in size and content.

Finally, there is an opportunity to circumvent existing paradigms in biology and induce vesicle fission through chemical methods that are apparently unrelated to the mechanisms of membrane fission in living organisms. In one fascinating example, Zhu et al. showed that simple, light-driven chemical reactions could have been involved in early cellular division and might be harnessed for synthetic biology applications.^[70] They demonstrated that ultraviolet illumination of filamentous fatty acid vesicles in the presence of thiols resulted in membrane pearling and division. They hypothesized that oxidized (but not reduced) thiols associated with the membrane, causing instabilities that lead to division. More recently, Miele et al. demonstrated fission of hybrid vesicles (i.e., vesicles containing both phospholipids and fatty acids) by increasing the alkalinity of the vesicle lumen through a simple enzymatic reaction.^[71] These researchers hypothesized that deprotonation of internal oleic acid molecules caused them to

dissociate from the membrane, thus lowering inner leaflet surface area. At the same time, the introduction of urea to the extravesicular environment initially caused osmotic shrinking of vesicles. Together, these effects were sufficient to cause division of giant unilamellar vesicles containing oleic acid. Further developing simple chemical methods for inducing division in lipid vesicles would be an interesting future direction of membrane mimetic chemistry.

2.4 Transport Across Membranes

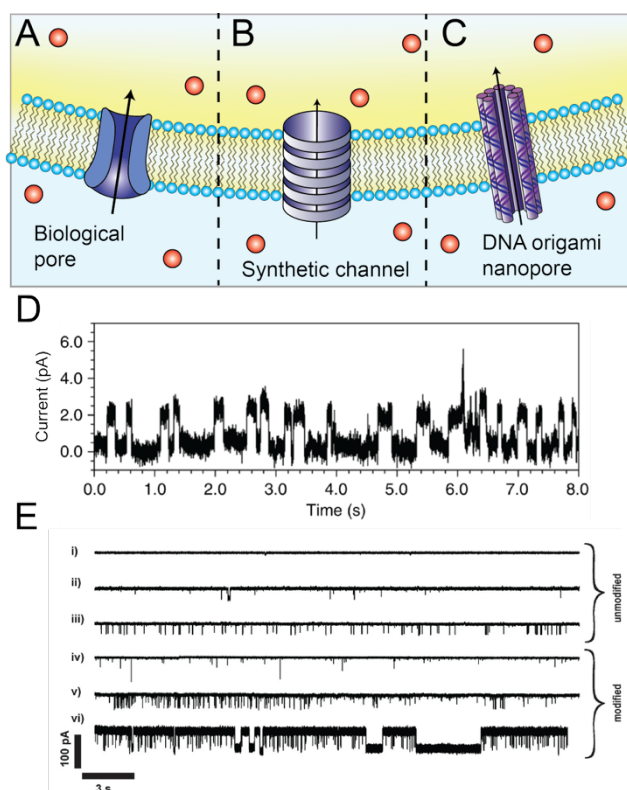


Figure 2.4 Methods for selectively permeabilizing artificial cell membranes.

(A) Reconstitution of biological pores can facilitate transport of solutes across phospholipid bilayers. (B) Synthetic mimics of biological pores can insert in membranes and permeabilize them to solutes. (C) DNA origami pores that can be rationally designed to insert into membranes creating pores of a specific diameter. (D) Current trace from a multi-block amphiphile inserted into a black lipid membrane (BLM) composed of DOPC using an applied voltage of 100 mV. (E) Representative current traces from electrical characterization of DNA nanopores performed on painted DPhPC (1,2-diphytanoyl-sn-glycero-3-phosphocholine) bilayers. [Panel D adapted with permission from ref 68. Copyright 2020, licensed under CC BY 4.0. Panel E adapted with permission from ref 70. Copyright 2012 The American Association for the Advancement of Science.]

Phospholipid membranes possess several advantages, including their biocompatibility and ability to maintain ion gradients. However, the limited permeability of phospholipid bilayer membranes also presents disadvantages for their use in artificial cells. Steady-state metabolism and growth of living cells requires that they continuously obtain material from the environment such as amino acids, nucleotides, and sugars, much of which does not permeate readily through phospholipid bilayers. Consequently, the comparatively high permeability of fatty acid membranes is attractive to synthetic biologists. However, reduced permeability is also necessary for living cells to maintain ion and metabolite gradients that are required for metabolism. For this reason, researchers have theorized that selective permeability of membranes arose gradually as protocells transitioned from fatty acid membranes to diacyl phospholipid membranes.^[37] Vesicle membranes that contain both fatty acid and phospholipid may therefore be appropriate platforms for some applications that require increased permeability. Demonstrating this concept, Zhou et al. recently examined ion permeability in hybrid phospholipid/fatty acid vesicles.^[72] Interestingly, they found that hybrid vesicles selectively retained potassium and excluded sodium, establishing transmembrane gradients for these ions in the absence of any protein-based pumps. This suggests that membrane permeability to ions in artificial cells can be modulated by simply adjusting membrane lipid content.

Modern cells evolved selectively permeable membranes by incorporating integral membrane proteins that are permeable to target molecules or ions. Ion gradients are maintained and metabolites are concentrated using ATP-dependent membrane-associated pumps. Thus, one of the most readily apparent strategies for affecting the

permeability of artificial membranes would involve the reconstitution of these membrane proteins in synthetic cells (Figure 2.4A). In general, reconstitution of membrane proteins in liposomes is challenging. This is traditionally done by solubilizing membrane proteins in detergent, adding phospholipid, and then removing excess detergent to generate proteoliposomes. This process can be time-consuming, and quantitative removal of detergent is challenging.^[73] Trace detergent molecules can inhibit the activity of some transmembrane proteins or can create defects in the membranes. Work in our group has explored reconstitution of membrane proteins in liposomes through alternative methods. For example, we have previously shown that several different integral membrane proteins, including G protein-coupled receptors (GPCRs), can be reconstituted in a functional form during de novo membrane synthesis.^[74] In this process, the detergents used to solubilize and purify the proteins are themselves reactive. Reaction with lysolipid analogs leads to artificial phospholipid membrane formation and spontaneous membrane protein reconstitution. The method does not require a large excess of reactive detergent, and the high yield of reaction leads to near-quantitative detergent depletion. More work is needed to determine whether proteins can be simultaneously expressed and reconstituted in membranes during de novo chemoenzymatic lipid synthesis using cell-free expression systems. In principle, in situ reconstitution of membrane proteins can be used to generate semi-permeable artificial cells. However, directionality of membrane proteins is difficult to control, which can impede the formation of proton gradients or membrane potentials in artificial cells using recombinant protein. This is a challenge that will need to be addressed if recombinant membrane proteins are to be used for this purpose. One potential solution was

demonstrated by Majumder et al., who showed that linker of nucleoskeleton and cytoskeleton (LINC) complexes could be reconstituted with a preferred functionality through cell-free expression of LINC proteins in the presence of a supported lipid bilayer.^[75]

Alternatively, phospholipid membranes can be permeabilized using non-protein reagents (Figure 2.4B,C). Ion channels and pores can permit diffusion of inorganic and organic solutes across hydrophobic membrane regions. Ion carriers, which sequester ions and facilitate their transport across the membrane, also serve this purpose.^[76] Significant efforts in the field of supramolecular chemistry have been dedicated to the development of synthetic channels, pores, and carriers for biological research.^[76] While molecules capable of increasing membrane permeability have long been identified, recent work has focused on creating molecules that allow transport with a preferred direction (e.g., outside to inside of vesicles) and that are responsive to external cues. Muraoka et al., for example, devised synthetic multi-pass ion channels that could be incorporated in an anisotropic manner into vesicular membranes (Figure 2.4D).^[77] They then demonstrated that these channels could be turned off and on through reversible ligand binding. Others have drawn inspiration from biological channels to design synthetic channels that are sensitive to light, pH, temperature, and mechanical force.^[78] An emerging area is the application of DNA origami to rationally create membrane channels. Simmel and co-workers have shown that DNA nanochannels in lipid membranes can show conductances and gating similar to natural ion channels and can discriminate between single DNA molecules (Figure 2.4E).^[79] These advancements will likely be crucial to the development of artificial cells and suggest that there are

alternatives to the reconstitution of transmembrane proteins for creating life-like membranes that are selectively permeable, responsive to external cues, and capable of establishing and maintaining gradients.

2.5 Lipid mesophases as synthetic organelles

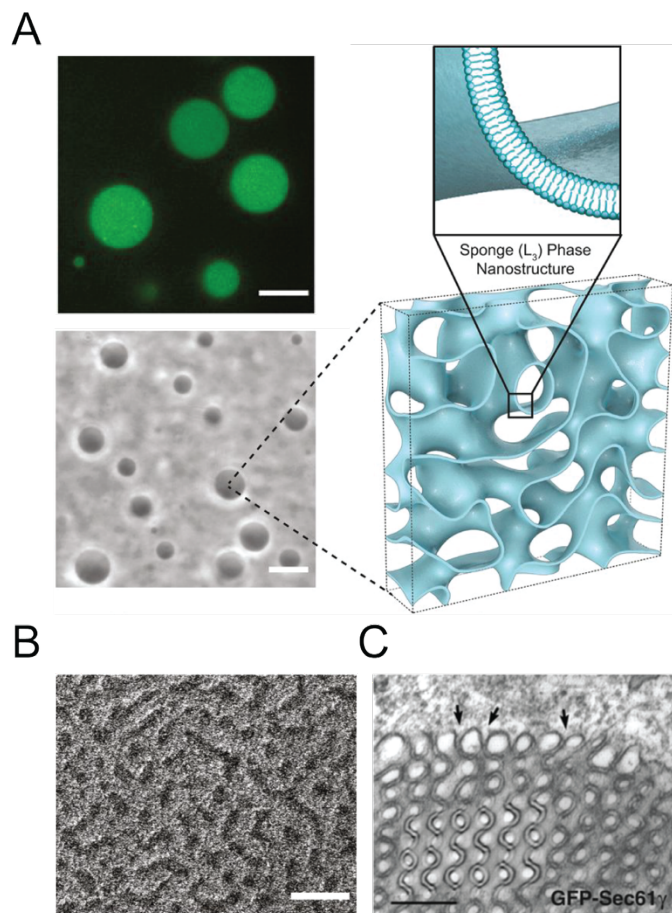


Figure 2.5 Cubic-phase lipid nanostructures as microcompartments for bottom-up synthetic biology.

(A) Lipid sponge droplet encapsulating Alexa Fluor 488-labeled cytochrome c oxidase. (B) Phase-contrast image of a typical droplet dispersion and an illustration of the droplets' porous, bilayer-rich, bicontinuous nanostructure. (C) Freeze-fracture cryogenic scanning electron microscopy (cryo-SEM) image of a dispersion showing the sponge phase morphology in lipid droplets from (B). [Panels A–C adapted with permission from ref 76.] (D) Distinct endoplasmic reticulum morphology induced by high levels of protein that are associated with formation of organized smooth endoplasmic reticulum. This morphology is consistent with a bicontinuous cubic lipid mesophase model described in ref 73. [Adapted with permission from ref 74. Copyright Rockefeller University Press 2003.]

While researchers have tended to employ lipid vesicles in bottom-up synthetic biology, few biological compartments resemble these spherical, lamellar structures. Living cells instead adopt a wide variety of shapes and sizes that are important for their

unique functions. Additionally, many organelles in living cells are highly interconnected rather than vesicular structures. These structures facilitate organellar functions that require membrane-dependent reactions and benefit from high membrane surface area, such as in the Golgi apparatus, the endoplasmic reticulum, and the mitochondria.^[80] Controlling the topology of artificial cells as well as their sub-compartments is a significant challenge in synthetic biology.^[17] The shape and size of lipid-based cellular compartments are in part determined by cytoskeletal elements. It would be quite difficult to reconstitute these networks in their entirety in artificial cells to precisely modify the shape and size of artificial organelles. However, properties of lipids themselves can impact physical properties of lipid compartments, including their morphology. The self-assembly of various natural and synthetic lipids into non-lamellar mesophases comprised of highly interconnected bilayer networks, such as cubic and sponge phases, is well-studied in vitro.^[81] Additionally, the extensive sponge-like morphology of the smooth endoplasmic reticulum (ER) has been documented in early electron microscopy studies, and cubic ER phases have been observed in cells, particularly those that are stressed, diseased, or virally infected (Figure 2.5D).^[82,83] Some lipid mesophases may therefore be appropriate models for organellar functions and could serve as platforms for bottom-up synthetic biology (Figure 2.5). Saw et al. recently showed that lipid substructures could be carefully tuned by simply adjusting relative amounts of lipids with different physical properties.^[83] These studies may be important in the development of artificial membranes and organelles for an artificial cell. Additionally, despite being observed in cells, non-lamellar lipidic structures *in cellulo* are underexplored and their functional roles are poorly understood. Modeling these compartments in vitro could help

elucidate their biological functions. It should be pointed out that it may not be necessary to identically mimic the observed structures, such as bicontinuous cubic phases, as alternative non-lamellar mesophases may have similar properties. Our group recently developed a novel biocompatible lipid sponge phase droplet capable of confining biological molecules and pathways with great efficiency (Figure 2.5A–C).^[84] Compartmentalization of protein into these bicontinuous sponge droplets is programmable, and remarkably high encapsulation efficiencies can be achieved. Past work has shown that various lipids such as gemini surfactants and natural glycolipids can form droplets consisting of non-lamellar lipid mesophases, and these systems are likely worth revisiting from the context of creating synthetic organelles.^[85,86] More work is needed to determine how mesophase lipid droplets could be coupled to synthetic cells to act as hubs for organization of biochemical reactions such as proteolysis and lipid synthesis.

2.6 Conclusion

All forms of life on Earth require lipid membranes. Living cell membranes are highly dynamic structures undergoing constant growth, degradation, division, and remodeling. By embracing membrane mimetic chemistry, there exists an opportunity to design and study synthetic membrane systems that possess dynamic properties rivaling those of living cellular membranes. Membrane mimetic chemistry was initiated to develop new chemistry by drawing inspiration from the compartmentalization of biomembranes. Faithful mimicry of biological phenomena was not the explicit goal. Instead, membrane-like environments such as micelles were used to organize reactants and catalyze chemical reactions. Over the past 40 years since these pioneering efforts,

there have been tremendous advances in fields such as chemical biology and synthetic biology. Coupled with the growth of sophisticated analytical techniques, there now exists an opportunity to expand the scope of membrane mimetic chemistry with the specific goal of using minimal chemical and biochemical systems to mimic the diverse functional phenomena encountered in biomembranes. Doing so may reveal fundamental phenomena related to cell membranes, may create new opportunities for applying membranes in biotechnology, and will accelerate progress in the rapidly developing research area of artificial cells.

2.7 Acknowledgements

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Chapter two, in full, is a reprint (with co-author permissions) of the material as it appears in the publication J. A. Vance, N.K. Devaraj “Membrane Mimetic Chemistry in Artificial Cells,” J. Am. Chem. Soc. 2021, 143, 22, 8223–8231. The dissertation author was the primary researcher and author of this material.

3 Targeted Detection of Phospholipid Aldehydes in Living Cells by Oxime

Ligation

3.1 Chapter abstract

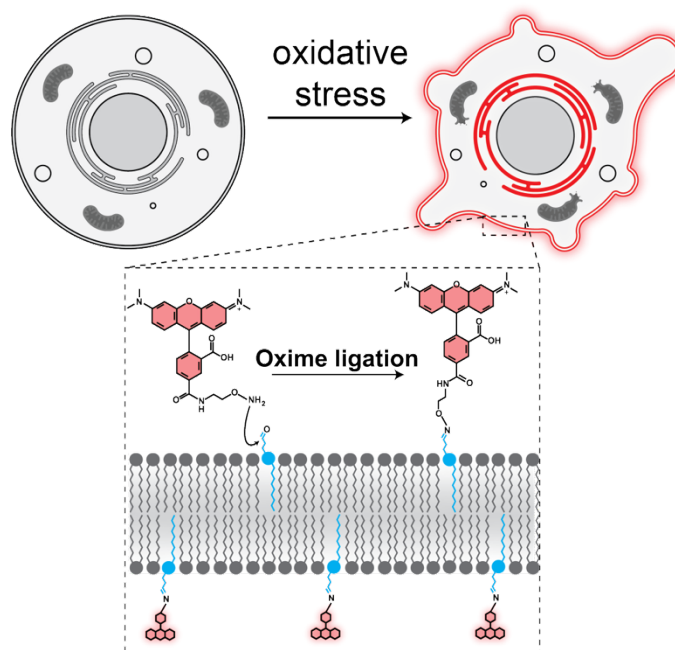


Figure 3.1 OxiLox overview.

Schematic of the reaction between aminooxy-TAMRA probes and truncated aldehyde lipids in oxidized cells.

Oxidatively damaged lipids play critical roles in numerous human pathologies, yet methods to directly identify these species within living cells remain limited due to their transient and low-abundance nature. Existing probes for imaging lipid oxidation often rely on indirect strategies, such as monitoring changes in the fluorescence intensity or color of oxidizable probes, which detect the presence of lipid peroxidation but not its direct chemical products. Among lipid oxidation products, aldehyde-containing lipids are particularly significant due to their heightened reactivity and strong links to disease pathology. Here, we introduce OxiLox (Figure 3.1), a method that leverages fluorescent hydroxylamine-based probes to enable direct, chemoselective tagging of aldehyde-containing lipids *in situ* within living cells via rapid oxime ligation. Using confocal

microscopy, we observe the subcellular localization of these oxidized lipids, highlighting prominent accumulation within perinuclear membranes, particularly under oxidative stress conditions induced by the ferroptosis activator RSL3. Coupled with high-resolution mass spectrometry, our method precisely identifies aldehyde lipid species, notably revealing the abundance of oxidized plasmenyl phosphatidylethanolamines in ferroptotic cells. We demonstrate that cellular labeling by hydroxylamine probes can be modulated by altering the expression of antioxidant enzymes such as aldose reductase (AKR1B1), underscoring the relationship between enzymatic antioxidant defenses and lipid aldehyde metabolism. Our work establishes OxiLox as a robust approach for direct, sensitive, and chemically precise detection of lipid aldehydes in live cells, offering critical new insights into the role of lipid oxidation in health and disease.

3.2 Introduction

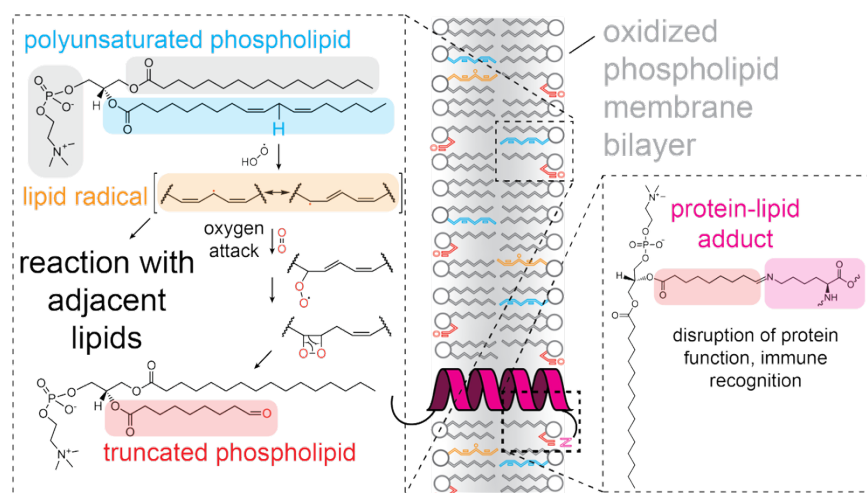


Figure 3.2 Graphic representation of the chemistry of oxidized lipid bilayers

Radical species abstract bis-allylic hydrogens from the polyunsaturated acyl chains (blue) of membrane phospholipids, leading to the formation of lipid radical species (orange), which can lead to a cascade of reactions resulting in acyl chain truncation (red). Reactive aldehyde-containing lipids can form adducts with proteins and other nucleophilic biomolecules in the cell (magenta).

Oxidative metabolism is fundamental to cellular energy production, yet inevitably generates reactive oxygen species (ROS) capable of damaging essential biomolecules.^[87,88] Among the most vulnerable targets of oxidative damage are polyunsaturated lipids in cellular membranes, whose bis-allylic hydrogens are prone to abstraction by ROS.^[1,89] This initial oxidative event triggers autocatalytic chain reactions driven by lipid peroxy radicals, perpetuating oxidative damage and disrupting membrane integrity and function (Figure 3.2). Under normal physiological conditions, cellular antioxidant defenses effectively neutralize ROS and oxidized lipids and prevent their toxic accumulation. However, such protective mechanisms can be overwhelmed during pathological conditions, resulting in extensive lipid peroxidation, cellular dysfunction, and death.^[90,91] Tracking and identifying the specific chemical products of lipid oxidation is thus critical to understanding the molecular underpinnings of oxidative stress-related diseases and for designing precise chemical interventions.^[92] Here we describe OxiLox, a method for directly targeting the reactive products of lipid peroxidation by oxime ligation. OxiLox enables detailed examination of lipid aldehyde subcellular localization through confocal microscopy and identification of oxidized lipid adducts via high-resolution mass spectrometry (Figure 3.1). Unlike prior fluorescence probes that only signal the presence of lipid peroxidation or mass-spectrometry approaches that require cell lysis before derivatization, our method chemically traps and identifies oxidized lipids *within* living cells.

Lipid peroxidation generates various products, including hydroperoxides, alcohols, epoxides, and aldehydes, each with distinct chemical properties and potential biological effects.^[93] The immune system recognizes many of these products as

damage-associated molecular patterns (DAMPs), promoting sterile inflammation and contributing to the progression of several chronic degenerative diseases.^[94–96] Among these species, aldehyde-containing lipids have garnered particular attention due to their pronounced reactivity and toxicity.^[97] The electrophilic nature of lipid aldehydes enables them to form covalent adducts with biomolecules, such as proteins and nucleic acids.^[98] These adducts can disrupt the function of key enzymes involved in antioxidant defense, contributing to the progression of membrane damage. Furthermore, lipid aldehydes disrupt the packing of lipids in membrane bilayers, contributing to a breakdown in membrane integrity.^[99] These findings position aldehyde lipids as potential primary drivers of the pathology associated with lipid oxidation, making them critical targets for investigation and intervention. Despite their importance, direct chemical detection and characterization of these lipid aldehydes in living cells remains challenging due to their transient nature, low abundance, and rapid cellular metabolism.

To address these chemical and biological challenges, we sought to leverage the chemoselectivity of oxime ligation, an efficient and stable condensation reaction between hydroxylamines and aldehyde/ketone moieties.^[8] Derivatization of biological carbonyls using α -nucleophile condensation reactions like hydrazone and oxime ligations has been well utilized as a bioconjugation strategy.^[100] In addition to their use for *in vitro* couplings, these reactions have been applied to capture oxidized carbonyls on biomolecules, including extracellular periodate-generated aldehydes on cell-surface glycans and allysine residues of extracellular matrix proteins in models of fibrosis.^[101,102] Indeed, hydrazine-containing compounds have been shown to inhibit foam cell formation, a key step in atherogenesis.^[103] And notably, hydrazine-based formaldehyde

probes have enabled live-cell and tissue imaging of endogenous formaldehyde via turn-on fluorescence upon hydrazone formation.^[104] Classically, slow carbonyl-condensation kinetics at neutral pH, combined with the low abundance and transient nature of cellular aldehydes, can restrict their cellular applications. Although imine-forming catalysts like aniline can accelerate these reactions, their propensity to induce oxidative stress and cytotoxicity remains a significant drawback.^[105] Compared to hydrazone ligation, which similarly forms a covalent bond with carbonyl groups, oxime ligation offers greater stability under mild conditions.^[106] These properties make oxime ligation particularly suitable for our application. Importantly, prior studies have indicated that oxime ligation proceeds rapidly and efficiently within lipid bilayers, overcoming the slow kinetics traditionally observed at neutral pH in aqueous environment.^[7]

Building on these past chemical insights, we hypothesized that fluorescent hydroxylamine probes could be optimized for the selective, rapid derivatization of lipid-derived aldehydes. We demonstrate that OxiLox forms an irreversible oxime linkage with endogenous lipid aldehydes in live cells, anchoring the fluorophore at the site of membrane damage for subcellular imaging by confocal microscopy. Existing probes report on earlier stages of lipid peroxidation through distinct mechanisms. C11-BODIPY senses lipid peroxidation by a ratiometric red-to-green fluorescence emission shift resulting from oxidation of the probe, with no formation of covalent adducts.^[107] A distinct approach is exemplified by the NBD-Pen probe, which detects lipid peroxidation through a redox-based turn-on fluorescence mechanism wherein direct reaction with lipid radicals relieves intramolecular quenching and activates fluorescence.^[108] A conceptually related strategy is exemplified by fluorogenic probes based on fluorogenic

radical-trapping antioxidants, which allow specific detection and quantification of lipid peroxyl radicals.^[109] By specifically targeting the aldehyde products that accumulate downstream of radical propagation, OxiLox delivers complementary chemical and temporal information that cannot be captured by either self-oxidizing dyes or radical-trapping probes. To generate live-cell compatible tools for labeling lipid aldehydes, we synthesized fluorescent hydroxylamine probes optimized for reactivity and evaluated their performance in model membrane systems and live cellular environments. Our results demonstrate selective labeling of oxidized lipids, particularly aldehyde-containing species, within cellular contexts. Moreover, hydroxylamine derivatives of oxidized lipids were analyzed directly using high-resolution mass spectrometry on lipid extracts from HT- 1080 cells undergoing ferroptosis, confirming probe specificity and reactivity. OxiLox can pinpoint which lipid peroxidation products account for the accumulation of fluorescence observed in cells undergoing oxidative stress. For instance, oxime adducts of plasmalogen-derived aldehydes (from plasmenyl phosphatidylethanolamines) increase significantly in ferroptotic cells, supporting previous suggestions that plasmalogens are highly oxidation-prone membrane components.^[9] We show that modulating the expression of a reductase involved in antioxidant defense influences both the accumulation of the hydroxylamine probe in living cells and the cells' susceptibility to toxicity caused by aldehyde lipids. These findings demonstrate that the extent of probe labeling correlates with the cellular capacity to metabolize reactive aldehyde lipid species, providing valuable physiological insights.

3.3 Results and Discussion

3.3.1 Design and synthesis of Aminoxy-TAMRAs for Selective Labeling of Phospholipid Aldehydes

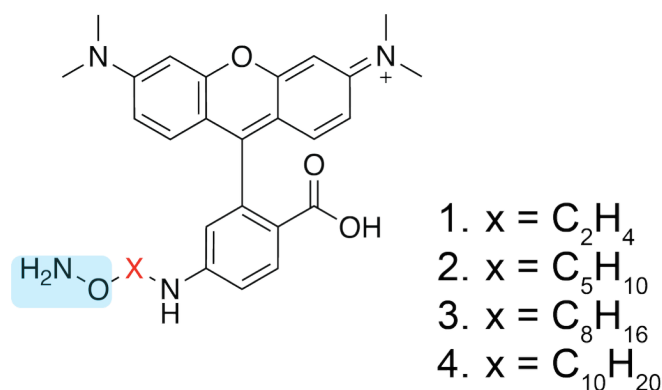


Figure 3.3 Chemical structures of aminoxy-TAMRA probes.

In total, four aminoxy-TAMRA probes were synthesized for this work. One of several alkyl linkers connects the TAMRA fluorophore to the hydroxylamine moiety.

To selectively label aldehyde-containing phospholipids, we designed a suite of tetramethylrhodamine (TAMRA) probes functionalized with hydroxylamine moieties. We hypothesized that tuning the hydrophobicity of the fluorescent hydroxylamine probe would be critical for optimal labeling of aldehyde-containing lipids. Molecular dynamics simulations suggest that the electrophilic aldehyde moiety in oxidized phospholipids can transiently protrude from the bilayer into the aqueous interface, thereby enhancing its accessibility for reaction.^[110] Given the interfacial position of the aldehyde, we hypothesized that optimal probe design would accommodate both hydrophobic and hydrophilic interactions. Excessive hydrophobicity risks nonspecific membrane association and elevated background fluorescence, whereas insufficient hydrophobicity could limit interaction with aldehydes at the bilayer surface. Furthermore, a highly hydrophilic probe would likely have increased off-target reactivity with small-soluble aldehydes such as formaldehyde and malondialdehyde. Thus, the best probes would likely strike a balance between rapid, selective labeling of lipid aldehydes in their native

environment while preserving aqueous solubility and cell permeability and allowing washout of the unreacted dye.

We chose to synthesize TAMRA derivatives as numerous previous studies have shown that TAMRA derivatives are suitable for live cell intracellular labeling, and if appropriately designed, can be washed out of cells, giving a high labeling signal over background.^[111,112] To further tailor the hydrophobicity of these probes, we appended hydroxylamine moieties onto the TAMRA scaffold via alkyl chains of varying lengths (Figure 3.3). Detailed synthetic procedures for aminooxy-TAMRA probes are provided in section **3.7 Structures and synthetic schemes**. In total, we chemically synthesized four aminooxy-TAMRAs with varying alkyl linker lengths: C2 (**1**), C5 (**2**), C8 (**3**) and C10 (**4**) (Scheme 2).

To evaluate chemical selectivity, all four aminooxy-TAMRA variants were challenged with a panel of biologically relevant carbonyls: membrane-embedded phospholipid aldehydes 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-(5'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC); soluble chain-truncation product 4-hydroxynonenal; formaldehyde; the oxidized cholesterol aldehyde atheronal A; and glucose, followed by liquid chromatography–mass spectrometry (LC-MS) analysis. Each aminooxy-TAMRA probe reacted preferentially and rapidly with phospholipid aldehydes POVPC and PONPC, while showing markedly lower conversion with the smaller or sterically distinct aldehydes (Figure S3.13-Figure S3.16). These results demonstrate that aminooxy-TAMRA dyes preferentially form oxime adducts with phospholipid aldehydes even in the presence of competing aldehydes.

3.3.2 Aminoxy-TAMRA probes form stable oxime adducts with polyunsaturated phospholipid-derived aldehydes in supported bilayers

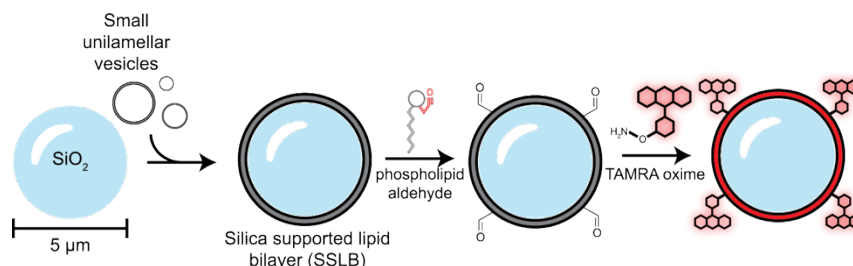


Figure 3.4 Schematic representation of *in vitro* testing of hydroxylamine probe retention on silica-supported lipid bilayers.

SSLBs are prepared by first incubating silica beads (labeled SiO₂) with small unilamellar vesicles such that the lipids adhere to the silica surface, forming a bilayer (silica supported lipid bilayer, SSLB). SSLBs are incubated with lipid aldehyde or control before reaction with aminoxy-TAMRA probes.

We sought to systematically evaluate the reactivity of our aminoxy-TAMRA probe variants toward lipid aldehydes under conditions that approximate the native hydrophobic environment of cellular membranes. We employed silica-supported lipid bilayers (SSLBs), in which phospholipid bilayers are assembled on a solid silica support (Figure 3.4).^[113] This configuration not only preserves the key biophysical properties of the lipid bilayer but also allows for consistent and efficient washing to remove unreacted dye. In contrast, vesicular systems are typically generated by rehydrating dried lipid films and are inherently heterogeneous, exhibiting significant variability in size, shape, and lamellarity.^[114] This variability complicates fluorescence quantification because the non-uniformity of vesicles can make direct comparisons between samples challenging. Using silica microspheres (~5 μm in diameter) patterned with lipids, we established a more controlled and uniform model system that enables precise assessment of probe reactivity and washability in a membrane-like context.

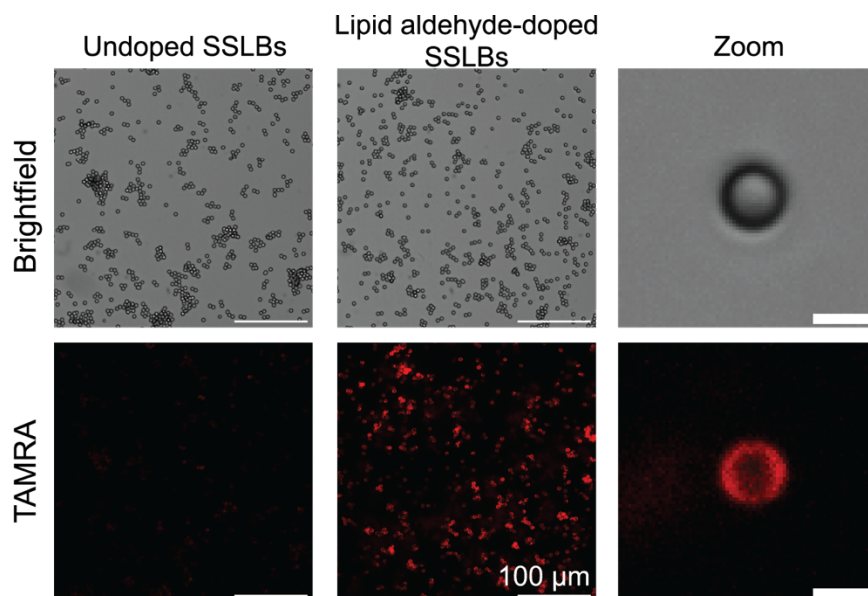


Figure 3.5 Representative images of lipid-aldehyde-doped SSLBs functionalized with an aminooxy-TAMRA fluorophore.

SSLBs were incubated with 1 μM 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC) before staining with aminooxy-TAMRA **1** for 30 minutes. After washing, SSLBs were imaged by confocal microscopy to determine extent of probe retention.

We generated our oxidized-lipid SSLB model by first depositing the natural phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) onto approximately 5×10^6 silica microspheres by incubation of the spheres in an aqueous 100 μL solution of 200 μM small unilamellar vesicles, followed by a brief incubation of the silica spheres in a 100 μL solution containing 1 μM 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC).^[30] Excess lipid was removed through multiple wash steps. We then exposed both doped and undoped SSLBs to our panel of TAMRA dyes (1 μM) in 10 mM phosphate buffer at physiological pH, followed by a thorough wash to remove unreacted dye (Figure 3.4). We then imaged the SSLBs using confocal fluorescence microscopy. By comparing fluorescence from doped versus undoped SSLBs, we could evaluate each probe's signal-to-background ratio. The signal in doped SSLBs arises from both specific oxime formation with aldehyde-containing lipids and

non-specific association with membranes. In contrast, fluorescence in undoped SSLBs reflects only non-specific membrane association. (Figure 3.5)

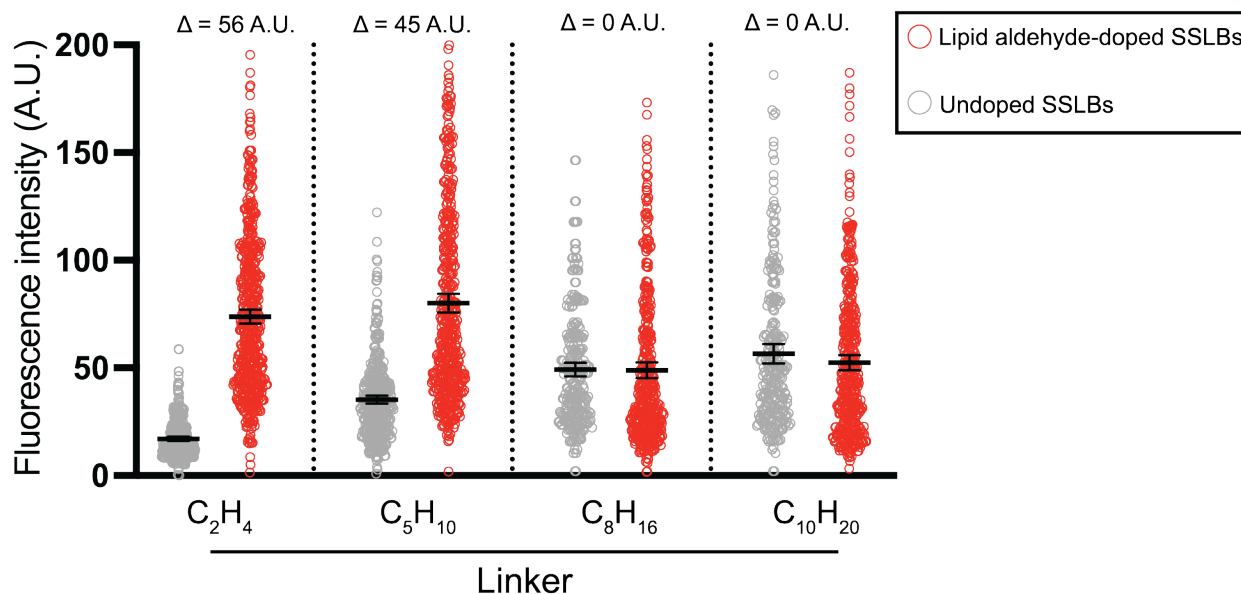


Figure 3.6 Quantification of SSLB fluorescence.

Lipid-aldehyde-doped (1 μ M) SSLBs were treated with aminooxy-TAMRA probes 1-4 (30-minute incubation, 1 μ M) to test the effect of alkyl linker length on probe reaction with lipid aldehydes in model bilayers. Data points represent the average fluorescence of individual SSLBs as calculated by mean pixel intensity. Black bars represent the median fluorescence, and error bars represent SEM, with $n = 500$ SSLBs measured per condition. Because SSLB samples undergo multiple wash steps, the plotted fluorescence reflects membrane-retained oxime adducts and unreacted aminooxy-TAMRA dye rather than total conversion. For comparison, the difference between the average fluorescence of doped and undoped beads for each aminooxy-TAMRA dye (**1-4**) is shown above the graph.

To screen our aminooxy-TAMRA probes, we incubated SSLBs with 1 μ M of each dye for 30 minutes at 37°C before washing and imaging by confocal microscopy. We observed fluorescent signal in the TAMRA channel on the surface of both doped and undoped SSLBs for all probes regardless of alkyl linker length (Figure 3.5), indicating a degree of nonspecific retention even after extensive washing. To quantitatively compare probe retention, we calculated the average pixel intensities of individual imaged SSLBs (Figure 3.6). Notably, nonspecific association with undoped SSLBs increased with longer alkyl linkers. In contrast, probes bearing two- and five-carbon linkers exhibited significantly greater retention on doped SSLBs compared to undoped SSLBs,

suggesting that covalent oxime formation with aldehyde lipids predominates over nonspecific interactions for these constructs. For the probes with longer alkyl chains, however, the fluorescence intensity of doped SSLBs was not statistically distinguishable from that of undoped, presumably due to high nonspecific binding as would be expected for more hydrophobic dyes.

We further characterized the reaction kinetics of OxiLox in our model system (Figure S3.17) and found that incorporation of aminooxy-TAMRA **1** into doped SSLBs increased rapidly, reaching optimal signal-to-background ratios after approximately one hour, while accumulation in undoped SSLBs was slower. Additionally, experiments with lower doping concentrations revealed that even at 0.1 μ M PONPC, SSLBs were more fluorescent than undoped controls (Figure S3.18). Finally, when SSLBs were doped with several structurally distinct aldehydes derived from oxidation of common membrane lipids (PONPC, atheronal A, 4-hydroxynonenal, and 4-hydroxydecenal), analysis of their fluorescence after incubation with aminooxy-TAMRA **1** revealed a preference for the truncated phospholipid aldehyde PONPC (Figure S3.19). Our results underscore the importance of carefully balancing hydrophobic and hydrophilic characteristics for probes designed to target oxidized membrane lipids.^[115] Based on these observations, we concluded that among the tested dyes, the TAMRA fluorophore bearing a two-carbon linker (aminooxy-TAMRA **1**) offered the best balance of hydrophobicity and reactivity for targeting phospholipid aldehydes.

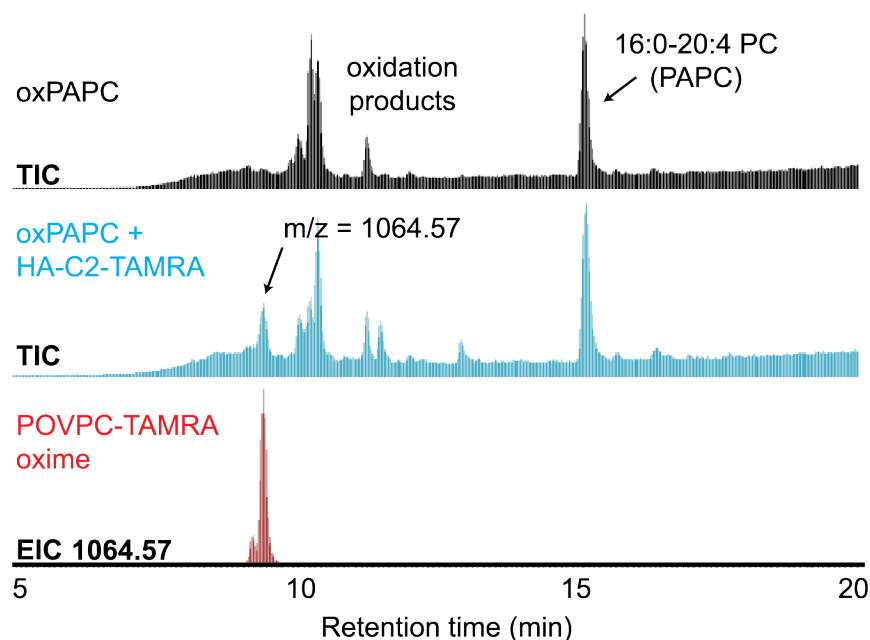


Figure 3.7 HR/MS analysis of lipid oxime from dried film.

1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (**PAPC**) was allowed to oxidize in air for 72 hours (**oxPAPC**) (top, black) and oxPAPC incubated with 1 μ M aminooxy-TAMRA **1** (middle, blue) for 1 hour after reconstitution in aqueous solution. The resulting reaction mixture was analyzed by LC/MS² to detect oximes formed in a complex mixture of oxidized lipids.

To further validate this probe in a more complex membrane environment, we prepared a dried film of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), a common polyunsaturated phospholipid in eukaryotic membranes, and allowed it to oxidize in air for 72 hours and then rehydrated it in 10 mM phosphate buffer (Figure 3.7). High resolution mass spectrometry combined with phase contrast microscopy confirmed that this procedure yielded multilamellar structures containing PAPC and various oxidative byproducts, including the truncated aldehyde phospholipid 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC), which was identified by matching retention time, exact mass, and MS/MS fragmentation to an authentic standard. Incubation of this oxidized lipid mixture with aminooxy-TAMRA **1** at 37 °C for 30 minutes resulted in the formation of an oxime adduct of POVPC, confirming that aminooxy-TAMRA **1** effectively labels phospholipid aldehydes in complex oxidized

membranes under physiologically relevant conditions. We thus employed aminoxy-TAMRA 1 probe in the OxiLox workflow for live cell imaging and mass spectrometry studies.

3.3.3 Reaction of aminoxy-TAMRA 1 with aldehyde lipids within living cell membranes

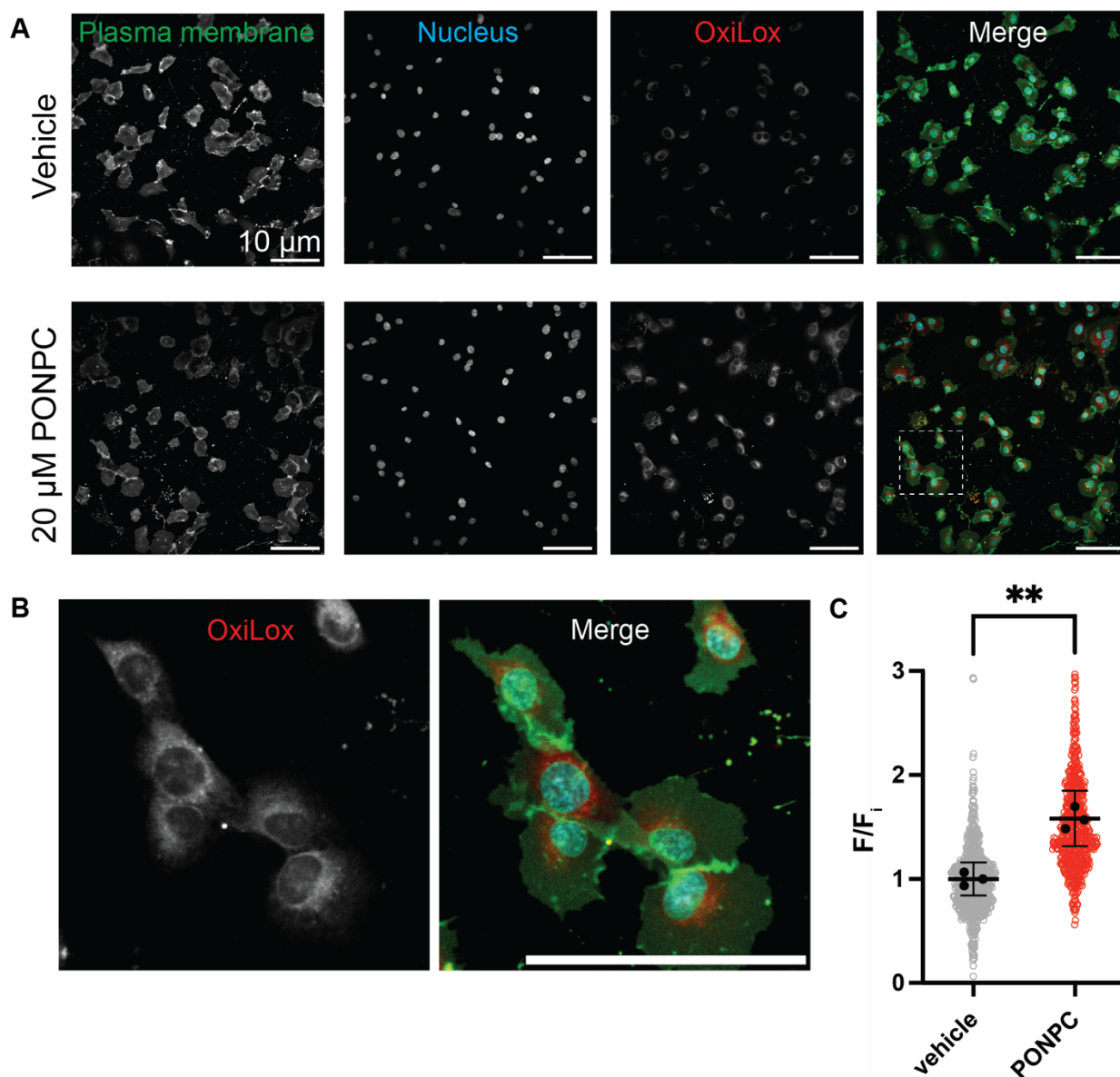


Figure 3.8 Reaction of aminoxy-TAMRA 1 with PONPC in live HT-1080 cells.

(A) Confocal micrographs of live HT-1080 fibrosarcoma cells incubated with 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC) (20 μ M) or vehicle for 30 minutes, followed by washing and staining with 1 μ M aminoxy-TAMRA 1 for 30 minutes. Scale bar = 20 μ m. (B) Zoomed in image of HT-1080 fibrosarcoma cells treated with PONPC and stained with aminoxy-TAMRA 1 showing prominent perinuclear staining. Scale bar = 20 μ m. (C) Quantification of fluorescence.

Having established aminooxy-TAMRA **1** as an optimized probe for OxiLox in model membrane systems, we next sought to validate its reactivity with oxidized lipids and in the complex environment of live cells. We selected HT-1080 fibrosarcoma cells for these experiments due to their well-characterized susceptibility to ferroptosis inducers and use as a model system in lipid oxidation and ferroptosis studies.^[91,116,117] These attributes make HT-1080 cells particularly suitable for investigating lipid peroxidation and reactive aldehyde production under controlled oxidative stress conditions. Initially, we assessed the ability of aminooxy-TAMRA **1** to label exogenously delivered lipid aldehydes in living HT-1080 cells. Cells were incubated with 20 μ M aldehyde-containing phospholipid PONPC for 30 minutes, followed by staining with 1 μ M aminooxy-TAMRA **1** for 30 minutes. Cells were co-stained with MemGlowTM 488 (Cytoskeleton Inc., cat. no. MG01) to delineate the cell surface prior to confocal microscopy imaging (Figure 3.8**A-B**). Quantitative analysis confirmed that PONPC-treated cells displayed higher fluorescence intensity compared to untreated control cells (Figure 3.8**C**). Cells that were treated first with the alkyl aldehydes pentanal and nonenal prior to staining did not exhibit significantly higher fluorescence than vehicle-treated cells (Figure S3.20). High-resolution mass spectrometry of lipid extracts confirmed significant formation of the expected PONPC–oxime adduct (Figure S3.21).

We also observed a moderate level of TAMRA fluorescence in untreated HT-1080 cells. Similar to observations made previously with SSLBs, this basal staining could reflect either non-specific hydrophobic interactions between aminooxy-TAMRA **1** and cellular membranes or the presence of endogenous lipid aldehydes present at steady state within the membranes, leading to specific oxime adduct formation and

retention of the dye. To address this, we rigorously tested multiple washing protocols designed to minimize nonspecific retention of unreacted dye and thus optimize the signal-to-background ratio. Despite a reduction in background staining with an optimized washing protocol, no procedure eliminated background staining completely, suggesting that some level of nonspecific membrane interaction or basal aldehyde presence is inherent to the live-cell application of this probe.

Nevertheless, the significant and reproducible increase in TAMRA fluorescence intensity following treatment with exogenous lipid aldehydes strongly supports the conclusion that aminooxy-TAMRA **1** effectively and specifically labels lipid aldehydes within cellular membranes. This finding validates the utility of the aminooxy-TAMRA **1** probe for directly visualizing aldehyde-containing lipid oxidation products under biologically relevant conditions.

3.3.4 OxiLox enables tracking of oxidized lipids in cells undergoing oxidative stress

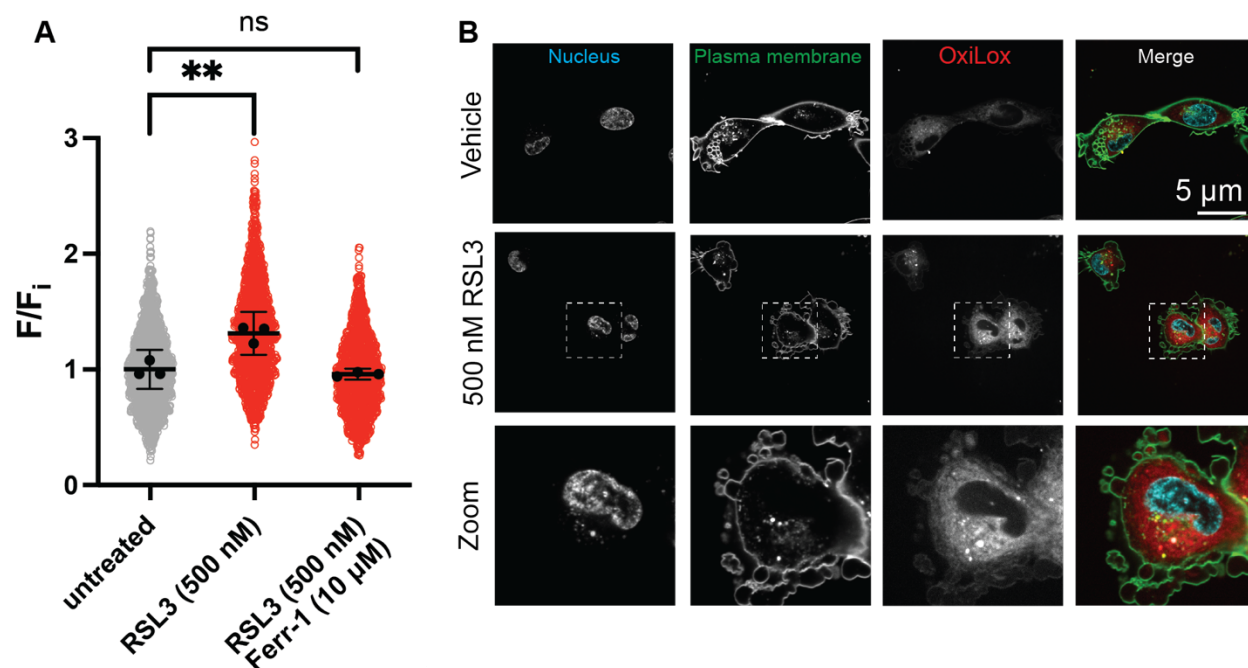


Figure 3.9 Confocal imaging of oxidized cells stained by OxiLox.

(A) Quantification of fluorescence of ferroptotic and control HT-1080 cells stained by OxiLox. Data representing fluorescence of individual cells from $n = 3$ biological replicates (individual wells of a 96-well plate), with ten images collected per replicate, is plotted. The mean fluorescence of all cells from single replicates is superimposed (bold black markers). These replicates were averaged, and their means compared by an unpaired t-test with Welch's correction (**, $P = 0.0061$, ns, not significant). **(B)** Confocal micrographs of HT-1080 fibrosarcoma cells treated for 2 hours with 500 nM RSL3, a potent inducer of ferroptosis, or vehicle, followed by 30 minutes staining with aminooxy-TAMRA **1**.

Next, we evaluated whether aminooxy-TAMRA probes could selectively detect and track endogenous lipid aldehydes generated during oxidative stress in cultured cells. HT-1080 fibrosarcoma cells were incubated for two hours with 500 nM RSL3, a potent ferroptosis inducer that specifically inhibits glutathione peroxidase 4 (GPX4), thereby triggering extensive lipid peroxidation.^[118] Following this treatment, cells were stained for 30 minutes with 1 μ M aminooxy-TAMRA **1** before imaging by confocal fluorescence microscopy.

Confocal images demonstrated significant increases in cellular TAMRA fluorescence upon RSL3 treatment compared to vehicle-treated controls (Figure 3.9A–

B). Co-treatment of RSL3-challenged HT-1080 cells with the lipophilic radical-trapping antioxidant ferrostatin-1 (fer-1) (10 μ M) abolished the RSL3-dependent increase in OxiLox staining (Figure 3.9A). Fluorescent labeling was clearly observed in multiple cellular compartments, prominently at the plasma membrane and perinuclear membranes. These observations align with previous findings that ferroptosis-driven lipid oxidation affects several membrane compartments rather than being restricted to a single subcellular location.^[107]

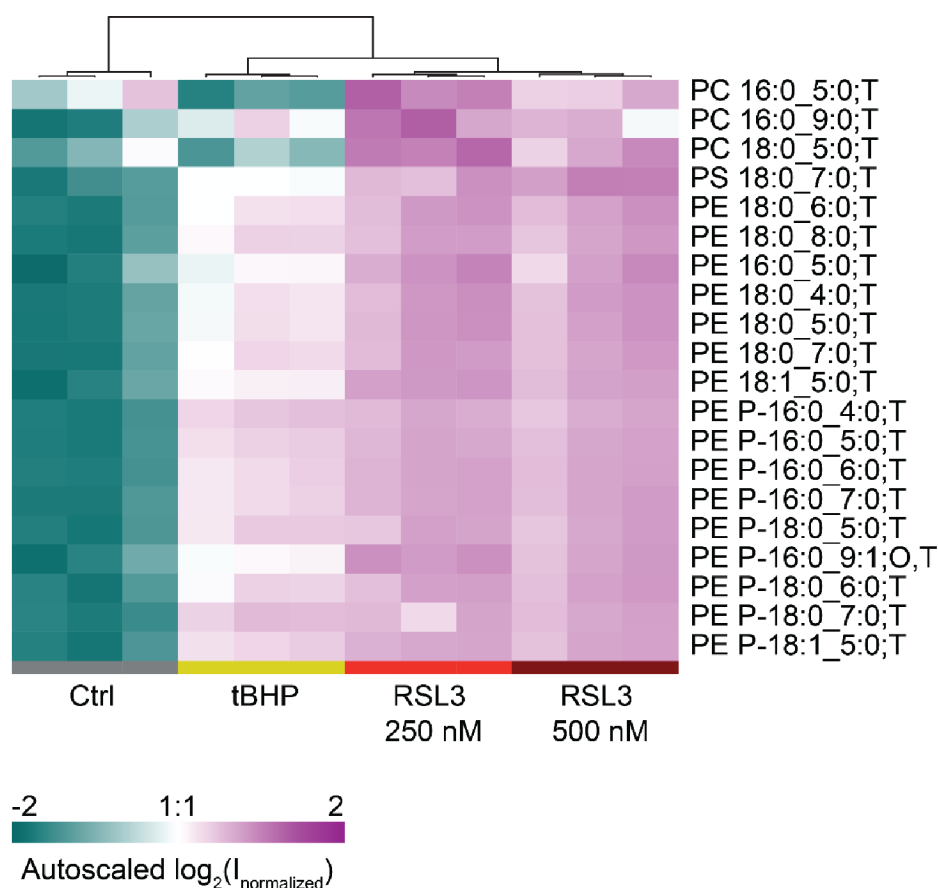


Figure 3.10 Relative quantification of phospholipid oximes detected in cell lysates.

Lipid extracts from untreated (Ctrl) and treated (100 μ M tert-butylhydroperoxide (TBHP), RSL3 250 nM, RSL3 500 nM) HT-1080 cells were analyzed by HRMS after staining with aminoxy TAMRA 1. Normalized intensities were $\log_2$ -transformed and autoscaled, and hierarchical clustering of individual samples was performed using Ward linkage agglomeration rule.

To identify the specific endogenous lipid aldehyde species responsible for the increased fluorescence, we conducted an extensive analysis by liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-MS/MS). Although the collective abundance of lipid aldehyde species is expected to increase under oxidative stress, the abundance of individual lipid aldehyde species can be very low. As such, our analytical approach sought to simultaneously detect and quantify multiple aldehyde species in parallel. First, we generated a comprehensive list of the most abundant polyunsaturated lipid-containing species known in the HT-1080 lipidome, encompassing phospholipids, triglycerides, and cholesteryl esters (Table S1). Using this lipid set, we computationally predicted the oxidized lipidome (oxilipidome) of HT-1080 cells with the LPPTiger2 software.^[119] The resulting list of oxidized lipids was filtered to retain only truncated oxo-containing products, for which we calculated the chemical formulas of the corresponding TAMRA oxime derivatives. This refined list was then used to generate an inclusion list for semi-targeted data-dependent acquisition (stDDA) by LC-MS/MS. Finally, pooled lipid extracts from RSL3-treated (500 nM and 250 nM), tertbutylhydroperoxide (TBHP)-treated, and untreated HT-1080 cells were analyzed to determine which of the predicted oxime derivatives were detectable in the samples.

The identities of detected TAMRA–lipid aldehyde adducts were rigorously confirmed by multiple analytical criteria: exact mass matching at MS1 level, characteristic shifts in retention times (TAMRA adducts consistently exhibited increased hydrophilicity relative to their corresponding free aldehyde lipids, Figure S3.22), and diagnostic tandem mass spectrometry (MS/MS) fragmentation patterns. Specific TAMRA core fragment ions of mass 386.1625 and 474.2024 were previously validated

using authentic oxime standards. They were consistently detected across the targeted lipid species, further substantiating their structural assignments (see accompanying file containing MS2 spectra for all detected compounds). Additionally, these identifications were cross-validated by detection in both positive and negative ionization modes. Detailed explanations of methods and spectra for these experiments are available in the supplementary information (Detailed experimental methods and MS2).

From this analysis, we selected the 20 most abundant TAMRA-labeled lipid aldehydes as representative target species for relative quantification by LC-MS in full scan MS acquisition mode. The relative abundance of these 20 lipid aldehyde–TAMRA adducts was then determined by comparing their peak intensities between treated cells (500 nM and 250 nM RSL3, 100 μ M TBHP) and untreated cells. Our results indicated that oxidized phospholipids, particularly plasmenyl phosphatidylethanolamines, were markedly increased in cells undergoing oxidative stress induced by RSL3 and TBHP, with the highest abundance of these species observed in cells treated with 500 nM RSL3 (Figure 3.10).

These observations confirm that oxime ligation to phospholipid aldehydes accounts at least partially for the increase in OxiLox labeling in treated HT-1080 cells observed by confocal microscopy. Additionally, it suggests that lipid oxidation during ferroptosis selectively enriches specific phospholipid aldehyde species rather than uniformly increasing all lipid aldehyde species equally. The enrichment of oxidized plasmenyl PEs may have broader implications, given their abundant presence in critical cellular membranes such as neuronal and cardiac tissues, suggesting these species could be key contributors to lipid peroxidation-associated pathologies.^[9] Indeed, genetic

studies have implicated plasmalogens as key drivers of ferroptosis.^[120] Comprehensive detection and quantification of all potential aldehyde–TAMRA adducts remains beyond current analytical capabilities due to both the extremely low abundance of individual oxidized lipid species and the enormous chemical diversity arising from lipid peroxidation. Nonetheless, the identification of multiple significantly increased aldehyde-containing lipids provides clear support for the validity and utility of this methodology. Collectively, our imaging and lipidomics experiments demonstrate that OxiLox is an effective method for selectively tracking and chemically characterizing endogenous lipid aldehydes generated under biologically relevant oxidative stress conditions.

3.3.5 Cells with elevated AKR1B1 expression exhibit reduced aminoxy-TAMRA labeling under oxidative stress

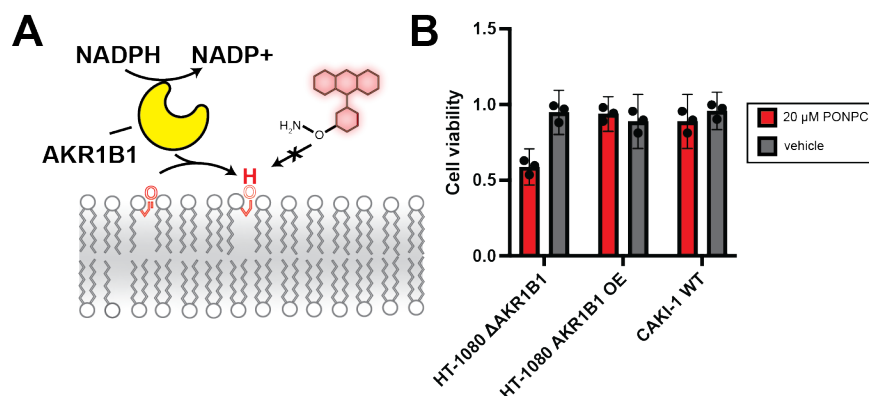


Figure 3.11 Schematic representation of reduction of phospholipid aldehydes and impact on cell viability.

(A) Schematic representation of reduction of phospholipid aldehydes by AKR1B1, leading to a loss of reactivity with aminoxy-TAMRA 1. (B) Viability of HT-1080 fibrosarcoma and CAKI-1 renal carcinoma cells treated with 20 μM 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC) or vehicle for 16 hours. The viability of HT-1080 fibrosarcoma cells with minimal expression (HT-1080 ΔAKR1B1, CRISPRi KO) or overexpression (HT-1080 pcDNA3.1-AKR1B1) AKR1B1 is compared to the viability of CAKI-1 renal carcinoma cells.

To evaluate the utility of OxiLox as a live-cell reporter of intracellular lipid aldehyde burden, we sought to determine whether probe labeling is modulated by endogenous antioxidant defenses, particularly enzymatic pathways that metabolize or detoxify aldehyde-containing lipids. Thus, we employed an aldose reductase enzyme,

AKR1B1, as a proof-of-concept target to validate whether OxiLox can report on endogenous aldehyde load and the activity of known aldehyde-metabolizing enzymes. This approach establishes a framework for future functional screening strategies to uncover additional, previously uncharacterized regulators of lipid electrophile metabolism.

AKR1B1 is an NADPH-dependent aldo-keto reductase enzyme implicated in the detoxification of reactive carbonyl species, including lipid-derived aldehydes.^[121] *In vitro* biochemical studies have shown that AKR1B1 efficiently reduces electrophilic lipid oxidation products (e.g., POVPC and PONPC) to their corresponding alcohols, thereby diminishing their reactivity and potential to form damaging adducts with proteins and nucleic acids (Figure 3.11A).^[121] AKR1B1 expression is induced by oxidative stress and is among the downstream targets of the transcription factor Nrf2, which orchestrates a broad cytoprotective program in response to redox imbalance, including oxidative stress caused by ischemia-reperfusion.^[122] However, the extent to which ischemic damage is mediated specifically by phospholipid-derived aldehydes remains unclear. Although the conversion of aldehydes to alcohols is expected to reduce electrophilicity, the downstream consequences of this transformation are a subject of ongoing research.^[123,124] Further, it remains unclear to what extent AKR1B1 activity influences the availability of these lipid aldehydes for covalent labeling by aminooxy-TAMRA **1** in living cells.

To investigate whether endogenous AKR1B1 activity modulates aminooxy-TAMRA **1** labeling of cells undergoing oxidative stress, we compared two human cancer cell lines with distinct basal levels of AKR1B1 expression. HT-1080 fibrosarcoma cells

exhibit relatively low expression of AKR1B1, while CAKI-1 renal carcinoma cells are reported to express high levels of AKR1B1.^[125] Given these intrinsic differences, we hypothesized that CAKI-1 cells would demonstrate an increased capacity to metabolize lipid aldehydes compared to HT-1080 cells, resulting in reduced aldehyde accumulation and, thus, decreased labeling by aminooxy-TAMRA **1** after ferroptosis induction.

To directly confirm whether AKR1B1 specifically modulates aldehyde metabolism, we genetically engineered HT-1080 cells to create models with either reduced or elevated AKR1B1 expression. We employed CRISPR interference (CRISPRi) to knock down AKR1B1 (generating the cell line HT-1080 Δ AKR1B1), and, separately, transiently overexpressed AKR1B1 using a plasmid encoding the full-length AKR1B1 protein (HT-1080 AKR1B1 OE cells). Detailed procedures, explanations of plasmids used in this study, and validations of knockdown efficiency and overexpression are provided in the supporting information (Detailed Experimental Methods, Figure S3.23 and Figure S3.24). Consistent with AKR1B1's proposed role in detoxification of phospholipid aldehydes, both CAKI-1 and HT-1080 AKR1B1 OE cells exhibited significantly decreased sensitivity to overnight treatment with 20 μ M PONPC compared to HT-1080 Δ AKR1B1 cells (Figure 3.11B). These results reinforce AKR1B1's role in detoxifying lipid aldehydes and promoting cellular resilience to oxidative damage. Given these clear functional differences in cell viability under treatment with PONPC, we next sought to determine whether these differences would be reflected in aminooxy-TAMRA **1** staining, thereby directly linking OxiLox labeling with live-cell enzymatic detoxification of lipid aldehydes.

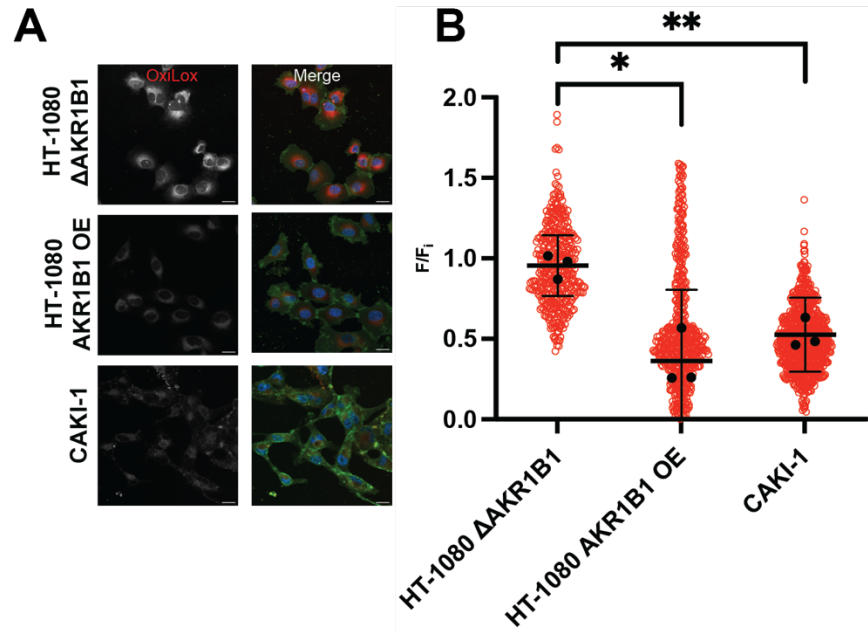


Figure 3.12 Representative confocal micrographs and quantification of treated cells overexpressing AKR1B1.

(C) Representative images of ferroptotic (500 nM RSL3-treated) HT-1080 cells with genetic manipulation of AKR1B1 expression compared to CAKI-1 cells after staining with aminooxy-TAMRA **1**. Cells were co-stained with MemGlow™ 488 (Cytoskeleton Inc, cat. #MG01) to stain plasma membranes and a DAPI nuclear stain (shown in merge). **(D)** Quantification of fluorescence of ferroptotic HT-1080 cells with genetic manipulation of AKR1B1 expression compared to CAKI-1 cells after staining with aminooxy-TAMRA **1**. Data representing fluorescence of individual cells from $n = 3$ biological replicates (individual wells of a 96-well plate), with ten images collected per replicate, is plotted. The mean fluorescence of all cells from single replicates is superimposed (bold black markers). These replicates were averaged, and their means compared by an unpaired t-test with Welch's correction (*, $P = 0.0169$, **, $P = 0.0039$).

To test this, we exposed HT-1080 (Δ AKR1B1, and AKR1B1 OE) and CAKI-1 cells to RSL3 (500 nM, 2 hours) to induce ferroptosis-driven lipid peroxidation, followed by incubation with aminooxy-TAMRA **1** (1 μ M, 30 min). Confocal microscopy and subsequent quantitative image analysis revealed significantly lower TAMRA fluorescence intensity in RSL3-treated CAKI-1 cells compared to similarly treated HT-1080 cells, suggesting a reduced accumulation of aldehyde-containing lipids due to higher AKR1B1 activity (Figure 3.12A,B). HT-1080 cells overexpressing AKR1B1 also displayed significantly diminished aminooxy-TAMRA **1** fluorescence following RSL3-induced oxidative stress relative to Δ AKR1B1 cells (Figure 3.12D). HT-1080 Δ AKR1B1

cells did not exhibit higher aminoxy-TAMRA **1** fluorescence, likely due to the already low basal levels of AKR1B1 expression in this cell line (data not shown).

Collectively, these findings provide robust evidence that intracellular OxiLox fluorescence intensity serves as a reliable indicator of lipid aldehyde accumulation and clearly demonstrates that this accumulation is significantly modulated by AKR1B1 enzymatic activity. Importantly, our study validates the utility of OxiLox for dissecting cellular lipid aldehyde metabolism under oxidative stress conditions. Furthermore, these results underscore the critical role of enzymatic aldehyde reduction pathways in controlling oxidative stress-induced lipid damage and associated cellular toxicity.^[126] Consequently, TAMRA labeling can serve as a powerful dual-purpose tool for identifying vulnerabilities to lipid electrophile stress and functionally profiling the cellular pathways responsible for lipid aldehyde detoxification, metabolism, and transport.

3.4 Conclusion

In this study, we introduce OxiLox, a method for imaging and directly profiling lipid aldehydes generated within living cells undergoing oxidative stress. Leveraging the chemoselectivity and stability of oxime ligation, we show that our aminoxy-TAMRA **1** probe enables subcellular visualization of lipid aldehyde localization and chemical identification by mass spectrometry. Using this approach, we imaged phospholipid aldehydes in perinuclear membranes and observed selective enrichment of specific oxidized lipid species in live cells undergoing ferroptosis. Notably, oxime derivatives of plasmalogen phosphatidylethanolamines were enriched in the membrane extracts of these cells, supporting their previously reported role in ferroptosis.

Although these probes represent a significant advancement for studying lipid oxidation dynamics, several limitations remain. We observed that TAMRA hydroxylamine dyes exhibit a degree of nonspecific association with cellular membranes, resulting in complicating background fluorescence and reducing the effective sensitivity of detection. Additionally, while our probes preferentially react with phospholipid-derived aldehydes, we cannot entirely exclude the possibility of reaction with other low-abundance cellular aldehydes, particularly under diverse pathological conditions. Further refinement of probe specificity will be important to enhance sensitivity and reduce background labeling.

Despite these limitations, OxiLox provides a robust chemical imaging platform with significant potential for future studies. For instance, using CRISPR-based genetic approaches, it could facilitate high-throughput screening of metabolic pathways regulating lipid aldehyde formation. Furthermore, OxiLox could be utilized to identify or characterize compounds that modulate lipid peroxidation, informing therapeutic strategies for diseases involving oxidative stress. Continued development and optimization of this methodology promises to significantly advance our understanding of lipid oxidation and its contributions to human disease.

3.5 Acknowledgements

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Chapter three, in full, is a reprint (with co-author permissions) of the material as it appears in the publication J. A. Vance, W. A. Chen, P. Nepachalovich, C. Knittel, M. Fedorova, N. K. Devaraj, *Angew. Chem. Int. Ed.* 2025, e202512578. <https://doi.org/10.1002/anie.202512578>. The dissertation author was the primary researcher and author of this material.

3.6 Detailed Experimental Methods

3.6.1 Determination of logP for compounds 1-4

LogP values were determined using the shake-flask method with *n*-octanol and water pre-saturated with each other. Compound **1-4** in DMSO was added to *n*-octanol/water mixture (final concentration = 250 µM, 0.5% DMSO v/v) in glass vials. The mixture was rotated at 25 °C for 1 h and phases were separated by centrifugation (3000 rpm, 5 min). Aliquots from each phase were diluted into MeOH, and injected and analyzed by HPLC (Eclipse Plus C8 column) in line with an Agilent 6100 Series Single Quadrupole MS (Agilent Technologies) with the flow rate of 1 mL/min. The linear

gradient of 95% MeOH was applied in the following 12 min. The absorbance of TAMRA was detected at 565 nm. LogP was calculated as:

$$\text{LogP} = \text{Log}_{10}\left(\frac{C_{\text{Octanol}}}{C_{\text{water}}}\right)$$

where C_{Octanol} and C_{water} are the concentrations in the respective phases.

3.6.2 *In Vitro Chemoselectivity Studies of Aminoxy-TAMRA probes*

2 μL of POPC (100 mM in CHCl_3), 1 μL of PONPC (20 mM in EtOH), POVPC (20 mM in EtOH), Atheronal A (20 mM in EtOH), 4-HNE (20 mM in EtOH) were placed in a 1/2 dram glass vial and the solvent was evaporated under vacuum. Then, 1 μL of glucose (20 mM in H_2O), glyoxal (20 mM in H_2O), formaldehyde (20 mM in H_2O) and 197 μL of PBS (pH 7.4) were added to the vial, which was then sonicated for 30 min. 1 μL of Aminoxy-TAMRA (20 mM in DMSO) was added to the mixer and shaken for 1 h at 37 $^{\circ}\text{C}$. Then, 198 μL of MeOH was added and vortexed. 10 μL of the mixture was then injected and analyzed by HPLC (Eclipse Plus C8 column) in line with an Agilent 6100 Series Single Quadrupole MS (Agilent Technologies) with the flow rate of 1 mL/min. The linear gradient of 95% MeOH was applied in the following 12 min. The absorbance of TAMRA was detected at 565 nm.

3.6.3 *Preparation of Silica Supported Lipid Bilayers (SSLBs)*

For preparation of silica supported lipid bilayers a 1 mg/mL solution in chloroform of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, cat. no. 850375) was dried to form a thin film on the surface of a small glass vial. Phospholipids were hydrated by addition of 10 mM phosphate buffer to yield a 1 mM DOPC suspension. The phospholipid suspension was then extruded 10 times through a 100

nm polycarbonate filter (Whatman, GE Healthcare Bio-Sciences) using an Avanti Mini-Extruder (Avanti Polar Lipids) to produce small unilamellar vesicles of uniform size.

To prepare silica beads, approximately 5×10^6 non-functionalized silica microspheres in aqueous suspension (Bangs Labs, 5 μ M size, cat no. SS05003) were added to 10 mM phosphate buffer to a total volume of 100 μ L. Silica microspheres were washed three times by centrifugation at 200 x g. On the final wash, 20 μ L of previously prepared DOPC SUV suspension was added and diluted with 80 μ L phosphate buffer. SSLB formation was induced by vortexing the solution at room temperature for 30 minutes. The SSLBs were then washed 3 times by centrifugation to remove excess lipids in phosphate buffer. Model oxidized SSLBs were prepared by treating SSLBs for 30 minutes with various concentrations and species of oxidized lipids, followed by washing by centrifugation.

3.6.4 In vitro hydroxylamine probe testing and imaging by spinning disk confocal microscopy

Prepared model SSLBs were incubated with various probes at several concentrations at 37°C with vortexing in phosphate buffer. After reaction, the SSLBs were washed by centrifugation and diluted into chambered coverslips for spinning disk confocal microscopy imaging.

3.6.5 Cell Culture

Wild-type HT-1080 fibrosarcoma and CAKI-1 renal carcinoma cells were obtained from the American Type Culture Collection (ATCC). HT-1080 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, ATCC, cat. no. 30-2002) and CAKI-1 cells were maintained in McCoy's 5A medium (ATCC, cat. no. 30-2007), both

supplemented with penicillin (50 units/mL), streptomycin (50 µg/mL) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco, cat. no. A5256801) at 37°C, 5% CO₂.

3.6.6 Plasmid Cloning for CRISPRi gRNA and AKR1B1 Overexpression

To generate a CRISPRi gRNA plasmid targeting *AKR1B1*, the 20 bp guide sequence in the backbone plasmids (pSG229) was replaced via standard molecular cloning techniques. The gRNA oligonucleotides were designed based on predictions from Weissman et al. and synthesized by Integrated DNA Technologies (IDT). pSG229 was amplified by PCR and linearized by restriction digestion followed by ligation to the 20 bp guide using NEB T4 ligase (New England Biosciences). After assembly, the construct was transformed into *E. coli* DH5α competent cells, and successful clones were identified by colony PCR and validated by whole plasmid sequencing (Plasmidsaurus). Both pmH0001 and pSG229, for repressor and guide expression, respectively, were generously provided by Sifeng Gu of the Alexis Komor lab at UC San Diego.

For *AKR1B1* overexpression, the full-length coding sequence optimized for mammalian expression was purchased from Twist Bioscience. The fragment was amplified with primers from IDT and cloned into a pcDNA3.1 vector backbone containing an mKate2 fluorescent reporter downstream of an IRES2 sequence using the NEB HiFi DNA Assembly Kit (New England Biolabs). After assembly, the construct was transformed into *E. coli* DH5α competent cells, and successful clones were identified by colony PCR and validated by whole plasmid sequencing.

3.6.7 Generation of AKR1B1 knockdown cell line

HT-1080 cells were engineered for CRISPR interference (CRISPRi) to suppress *AKR1B1* expression. Initially, cells were transduced with a lentiviral vector encoding the

dCas9 repressor construct. A clonal population was isolated by single-cell fluorescence-activated cell sorting (FACS) to ensure uniform dCas9 expression. Next, cells were transduced with a second lentiviral vector carrying a guide RNA (gRNA) targeting AKR1B1 and a clonal population was isolated by FACS to ensure uniform knockdown. Knockdown efficiency was confirmed by quantitative PCR (qPCR), demonstrating suppression of AKR1B1 expression.

3.6.8 Treatment of HT-1080 or CAKI-1 Cells with Exogenous PONPC

HT-1080 cells were seeded at 10,000 cells per well in 96-well glass-bottom plates (Ibidi, cat. no. 89626) and allowed to adhere overnight. The following day, cells were washed once with Hank's Balanced Salt Solution (HBSS) (Gibco, cat. no. 14025092) and treated with 20 μ M PONPC for 5 minutes. After a brief wash with HBSS, cells were incubated for 30 minutes with 1 μ M HA-C2-TAMRA (a TAMRA-conjugated hydroxylamine dye) to label oxidized lipids. Unbound dye was removed by three washes with Fluorobrite DMEM (Gibco, A1896701), each wash including a 5-minute incubation. Finally, cells were co-stained with Hoechst nuclear dye (Invitrogen, cat. no. R37605) and an Alexa-Fluor 488 conjugated membrane stain (Cytoskeleton, cat. no. MG01) to mark cell bodies, facilitating confocal imaging and quantifying per-cell fluorescence. All incubations and imaging were carried out in an incubator at 37°C, 5% CO₂.

3.6.9 Treatment of HT-1080 and CAKI-1 Cells with RSL3 for Confocal Imaging

HT-1080 and CAKI-1 cells were plated in triplicate at 10,000 cells per well in 96-well glass-bottom plates (Ibidi, cat. no. 89626) and allowed to adhere overnight as described above. The following day, cells were treated with 500 nM RSL3 (an inducer of ferroptosis, Med Chem Express, cat. no. HY-100218A) in Fluorobrite DMEM for 2 hours

at 37 °C. After treatment, cells were stained with Hoechst nuclear dye and an Alexa-Fluor 488 conjugated membrane stain (Cytoskeleton, cat. no. MG01) to label cell bodies. Following staining, cells were washed three times with Fluorobrite DMEM, with each wash involving a 5-minute incubation, to ensure removal of excess dye and RSL3 before confocal imaging.

3.6.10 Confocal Imaging

Images were acquired using a Nikon W1 SoRa spinning disk confocal microscope mounted on an Eclipse Ti2-E body. The system features an automated XY stage with a Queensgate piezo drive (600 μ m travel) and a Tokai Hit full enclosure that maintains a controlled environment at 37 °C with regulated CO₂ and humidity. Illumination was provided by a LUNF-XL laser box offering excitation wavelengths of 405, 445, 488, 514, 561, 594, and 640 nm. Image acquisition was automated using Nikon's NIS-Elements software (version 5.42.02 with the High Content Analysis package). For each well, 10 images per replicate were captured at predetermined positions arranged in a spiral pattern originating from the center of the well, ensuring consistent sampling across the plate in the same focal plane.

3.6.11 Image Analysis and Quantification

Images were analyzed using the open source CellProfiler software. For each image, background subtraction was performed by averaging the intensity of non-cell-containing regions and subtracting the value from the entire image, and images were cropped to remove edge artifacts. Nuclei were identified in the DAPI channel using the "Identify Primary Objects" module, while cell bodies were delineated in the AF-488 channel using the "Identify Secondary Objects" module. The average per cell

fluorescence was calculated in the TAMRA channel for each condition across three replicates. Data were normalized and statistical comparisons between conditions were then conducted using standard statistical tests.

3.6.12 LC/MS-MS

Reversed-phase ultra-high-performance LC (RP-UHPLC) was performed on a Vanquish Horizon (Thermo Fisher Scientific) equipped with an Accucore C30 column (150 × 2.1 mm, 2.6 μm, 150 Å, Thermo Fisher Scientific). 2 μL (positive mode) or 4 μL (negative mode) of pooled group-specific sample, or 8 μL of individual samples (positive mode, quantification) injected onto the column was separated by gradient elution with solvent A (CH₃CN:H₂O, 1:1, v/v) and B (*i*-PrOH:CH₃CN:H₂O, 85:10:5, v/v/v) both containing 5 mM HCOONH₄ and 0.1 vol % HCOOH. Separation was performed at 50°C at a flow rate of 0.3 mL/min using the following gradient: 0-5 min — 10-80% B, 20-24 min — 80–95% B, 24-27 min — 95-100% B, 27-32 min — 100% B (isocratic), 32-32.1 min — 100-10% B, 32.1-40.0 min — 10% B (isocratic, column re-equilibration).

Mass spectrometry was performed on Thermo Scientific Orbitrap Exploris 240 (Thermo Fisher Scientific) equipped with a heated electrospray ionization (HESI) source with an EASY-IC unit for lock mass correction and operated with the following global HESI parameters: sheath gas 40 arbitrary units, auxiliary gas 10 arbitrary units, sweep gas 1 arbitrary units, spray voltage 3.5 kV (positive mode) or 2.5 kV (negative mode), ion transfer tube temperature 300°C, vaporizer temperature 370°C, S-lens RF level 35%, EASY-IC lock mass correction was set to RunStart.

For identification of TAMRA derivatives, MS data was recorded in positive and negative ionization modes with the instrument operating in semi-targeted data

dependent acquisition (stDDA) mode with 6 MS/MS scans per cycle. Full scans (MS1) had the following settings: Orbitrap resolution 60000 at m/z 200, scan range m/z 400-900 and 900-1400 (positive mode) or 450-950 and 950-1450 (negative mode), absolute AGC value set to standard, maximum injection time set to auto, 1 microscan.

stDDA MS2 (ddMS2) scans had the following settings: 1.5 m/z precursor selection isolation window, Orbitrap resolution 60000 at m/z 200, stepped higher-energy collisional dissociation (HCD) at normalized collision energies of 17-27-37% (in both positive and negative modes), absolute AGC value $1 \cdot 10^5$, maximum injection time 200 ms, 2 microscans. The following filters were applied prior to ddMS2 scans: dynamic exclusion after 5 times if occurring within 6 s, exclusion duration 5 s, mass tolerance ± 5 ppm, isotope exclusion; allowed charge state 1-3 (positive mode) or 1 (negative mode); targeted mass inclusion using the mass list of *in silico* predicted TAMRA derivatives, inclusion mass tolerance ± 5 ppm. Data was acquired in profile mode.

To predict potential TAMRA derivatives, an *in silico* oxidation of the most abundant PUFA-containing lipids in HT1080 cells (based on in-house data) was performed using LPPtiger2 software. The lipid panel included 8 cholesteryl esters, 8 diacylglycerols, 13 diacylphosphatidylcholines, 6 alkylacylphosphatidylcholines, 8 diacylphosphatidylethanolamines, 9 alkenylacylphosphatidylethanolamines, 6 diacylphosphatidylglycerols, 5 diacylphosphatidylinositols, 4 diacylphosphatidylserines, and 18 triacylglycerols (Table S1). Oxidation parameters were set to a maximum of 2 sites per lipid, allowing up to 4 oxygen atoms, including up to 2 hydroxyl (OH), 2 keto, and 1 hydroperoxy (OOH) group. The resulting list of oxidized lipids (Table S2) was filtered to retain only truncated products containing an omega-oxo group. The elemental

compositions of the corresponding TAMRA derivatives were then calculated and used to generate an inclusion list for stDDA acquisition.

For relative quantification of TAMRA derivatives, full MS acquisition was performed in positive ionization mode with the following instrument settings: Orbitrap resolution 60000 at m/z 200, scan range m/z 480-700 within 5-17 min, AGC target value set to standard, maximum injection time set to auto, 1 microscan. Data was acquired in profile mode.

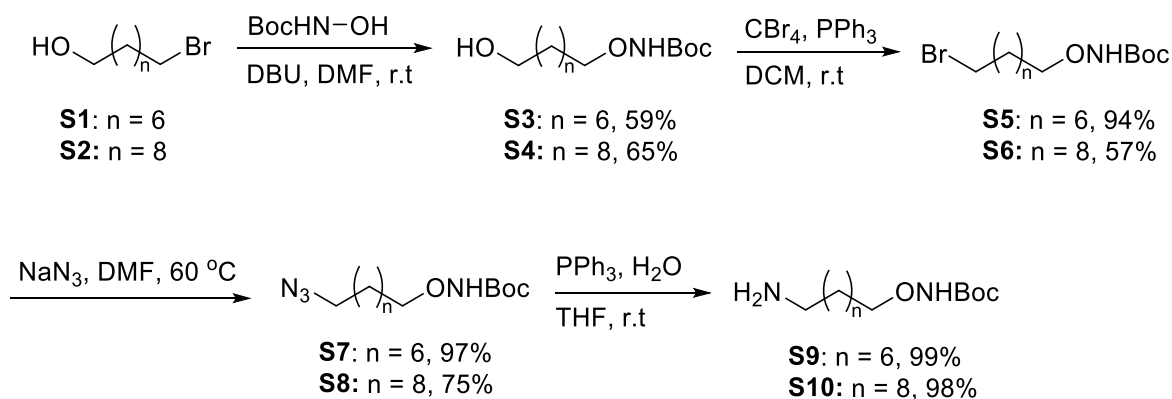
For sample normalization, total protein content was determined using Pierce™ BCA Protein Assay Kit (#23227, Thermo Scientific) following the manufacturer guidelines.

3.6.13 Cell Viability and Toxicity Assays

HT-1080 cells were seeded at 10,000 cells per well in 96-well plates and allowed to adhere overnight. Cells were then either transiently transfected with pcDNA3.1 encoding AKR1B1 using Transit transfection reagent or left untransfected as controls. Following transfection, cells were treated with 20 μ M PONPC or an equivalent volume of vehicle for 16 hours. Viability was assessed using the CellTiter-Glo luminescent assay (Promega) according to the manufacturer's instructions, and luminescence was quantified with a microplate reader. All conditions were performed in triplicate, and viability data were normalized to the respective vehicle-treated controls.

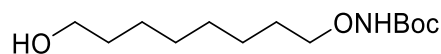
3.7 Structures and synthetic schemes

3.7.1 Synthesis of linkers S9 and S10



Scheme 1: Synthesis of linkers **S9** and **S10**.

3.7.2 Preparation of S3

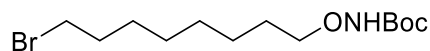


Supplementary Structure **S3**

DBU (1.09 g, 1.07 mL, 5 Eq, 7.17 mmol) was added to a mixture of *tert*-butyl hydroxycarbamate (382 mg, 2 Eq, 2.87 mmol) and 8-bromooctan-1-ol (300 mg, 1 Eq, 1.43 mmol) in DMF (5 mL). The mixture was allowed to stir for 24 h and then dissolved in DCM, and the resulting solution was washed sequentially with 1 N HCl and brine. The organic layer was dried (MgSO_4), filtered, and concentrated. The resulting yellow oil was purified by column chromatography (Hex:EtOAc = 1:6, silica gel) to provide **S3** (219.61 mg, 840.26 μmol , 58.6 %) as a colorless oil.

S3 TLC: (Hex /EtOAc 1:1 v/v) $R_f = 0.5$; ^1H NMR (400 MHz CDCl_3): δ 3.75 (td, $J = 6.7, 1.5$ Hz, 2H), 3.54 (td, $J = 6.7, 1.5$ Hz, 2H), 1.51-1.55 (m, 2H), 1.46-1.49 (m, 2H), 1.40 (s, 9H), 1.24 (m, 8H); ^{13}C NMR (100 MHz CDCl_3): δ 157.1, 81.5, 76.8, 62.7, 32.6, 29.3, 29.1, 28.2, 28.0, 25.8, 25.6.; HRMS (m/z): $[\text{Na}]^+$ calcd. for $\text{C}_{13}\text{H}_{27}\text{NO}_4$: 284.1832; found, 284.1834.

3.7.3 Preparation of S5



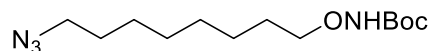
Supplementary Structure **S5**

PPh_3 (201 mg, 2 Eq, 765 μmol) and **S3** (100 mg, 1 Eq, 383 μmol) were dissolved in anhydrous DCM (1 mL) under an argon atmosphere. Pyridine (60.5 mg, 61.6 μL , 2 Eq, 765 μmol) and CBr_4 (254 mg, 2 Eq, 765 μmol) were added, and the reaction mixture stirred at room temperature for 24 h. The mixture was poured into water, extracted with DCM and the combined organic extracts were dried (MgSO_4), filtered, and concentrated. The product was purified by column chromatography (EtOAc/hexane, 1:20, silica gel) to provide **S5** (117 mg, 361 μmol , 94.3 %) as a colorless oil.

S5: TLC (n-hexane / EtOAc, v/v 5:1), $R_f = 0.5$. ^1H NMR (400 MHz, CDCl_3): δ 3.71 (t, $J = 6.7$ Hz, 2H), 3.28 (t, $J = 6.9$ Hz, 2H), 1.68-1.76 (m, 2H), 1.46-1.53 (m, 2H), 1.36 (s, 9H), 1.19-1.32 (m, 8H); ^{13}C NMR (100

MHz, CDCl₃): δ 157.0, 81.5, 76.7, 34.0, 32.8, 29.2, 28.6, 28.2, 28.1, 28.0, 25.8.; HRMS (m/z): [Na]⁺ calcd. for C₁₃H₂₆BrNO₃, 346.0988; found, 346.0987.

3.7.4 Preparation of S7

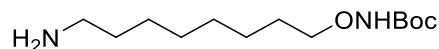


Supplementary Structure S7

Sodium azide (46.93 mg, 2 Eq, 722 μ mol) was added to a mixture of **S5** (117 mg, 1 Eq, 361 μ mol) in DMF (1 mL) and stirred for 24 hours at 60 °C. After the reaction was complete, reaction mixture was extracted with water and ethyl acetate. The organic layer was concentrated and purified by column chromatography (Hex/EtOAc = 20:1, silica gel) to provide **S7** (89 mg, 289 μ mol, 80%) as a colorless oil.

S7: TLC (n-hexane / EtOAc, v/v 5:1), R_f = 0.6. ¹H NMR (400 MHz, CDCl₃): δ 3.90 (t, *J* = 6.6 Hz, 2H), 3.32 (t, *J* = 7.0 Hz, 2H), 1.62-1.71 (m, 4H), 1.54 (s, 9H), 1.37-1.43 (m, 8H); ¹³C NMR (100 MHz, CDCl₃): δ 157.0, 81.6, 76.8, 51.5, 29.3, 29.0, 28.8, 28.3, 28.0, 26.6, 25.8.; HRMS (m/z): [Na]⁺ calcd. for C₁₃H₂₆N₄O₃, 309.1897; found, 309.1899.

3.7.5 Preparation of S9

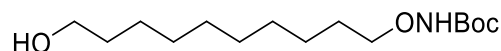


Supplementary Structure S9

PPh₃ (151.6 mg, 2 Eq, 578 μ mol) was added to a mixture of **S7** (89 mg, 1 Eq, 289 μ mol) and water (52 mg, 10 Eq, 2.89 mmol) in THF (1 mL) and stirred for 24 hours at 25 °C. After the reaction was complete, reaction mixture was concentrated and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **S9** (198.5 mg, 688.2 μ mol, 96 %) as a colorless oil.

S9: TLC (DCM / MeOH / NH₄OH, v/v 4:1:0.5), R_f = 0.5. ¹H NMR (400 MHz, MeOD): 3.74 (t, *J* = 6.6 Hz, 2H), 2.69 (t, *J* = 7.4 Hz, 2H), 1.59-1.47 (m, 4H), 1.43 (s, 9H), 1.33-1.38 (m, 8H); ¹³C NMR (100 MHz, MeOD): δ 157.7, 80.4, 76.0, 48.5, 40.6, 30.8, 29.1, 29.0, 27.7, 27.3, 26.4, 25.6.; HRMS (m/z): [H]⁺ calcd. for C₁₃H₂₈N₂O₃, 261.2173; found, 261.2176.

3.7.6 Preparation of S4

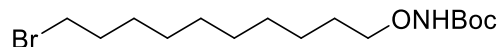


Supplementary Structure S4

Following the above general method for **S3**, compound **S2** (600 mg, 2.53 mmol) gave **S4** (474 mg, 1.64 mmol, 64.7%) as a colorless oil.

S4: (n-hexane /EtOAc 1:1 v/v) R_f = 0.4. ¹H NMR (400 MHz CDCl₃): δ 3.75 (t, *J* = 6.7 Hz, 2H), 3.55 (t, *J* = 6.7 Hz, 2H), 1.60-1.44 (m, 4H), 1.41 (s, 9H), 1.18-1.29 (m, 12H).; ¹³C NMR (100 MHz CDCl₃): δ 158.7, 81.6, 76.8, 62.8, 32.5, 29.5, 29.4, 29.4, 28.2, 28.0, 25.8, 25.7. HRMS (m/z): [Na]⁺ calcd. for C₁₅H₃₁NO₄: 312.2145; found, 312.2147.

3.7.7 Preparation of S6

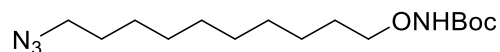


Supplementary Structure **S6**

Following the above general method for **S5**, compound **S2** (474 mg, 1.64 mmol) gave **S6** (330 mg, 0.94 mmol, 57.2%) as a colorless oil.

S6: TLC (n-hexane / EtOAc, v/v 5:1), R_f = 0.6. ^1H NMR (400 MHz CDCl_3): δ 7.29 (br, 1H), 3.78 (t, J = 6.7 Hz, 2H), 3.36 (t, J = 6.9 Hz, 2H), 1.83-1.76 (dt, J = 14.7, 6.9 Hz, 2H), 1.56 (dt, J = 14.8, 6.6 Hz, 2H), 1.43 (s, 9H), 1.20-1.38 (m, 12H).; ^{13}C NMR (100 MHz CDCl_3): δ 157.0, 81.5, 76.8, 34.1, 32.8, 29.4, 28.7, 28.2, 28.2, 28.0, 25.9.; HRMS (m/z): $[\text{Na}]^+$ calcd. for $\text{C}_{15}\text{H}_{30}\text{NO}_3\text{Br}$: 374.1301; found, 374.1301.

3.7.8 Preparation of S8

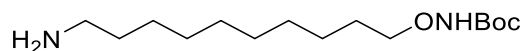


Supplementary Structure **S8**

Following the above general method for **S7**, compound **S6** (330 mg, 0.94 mmol) gave **S8** (221 mg, 0.7 mmol, 75%) as a colorless oil.

S8: TLC (n-hexane / EtOAc, v/v 5:1), R_f = 0.6. ^1H NMR (400 MHz CDCl_3): δ 7.41 (br, 1H), 3.75 (t, J = 6.7 Hz, 2H), 3.17 (t, J = 7.0 Hz, 2H), 1.53 (ddt, J = 15.6, 8.8, 6.7 Hz, 4H), 1.41 (s, 9H), 1.18-1.32 (m, 12H).; ^{13}C NMR (100 MHz CDCl_3): δ 157.0, 81.3, 76.7, 51.4, 29.4, 29.1, 28.8, 28.2, 28.0, 26.7, 25.8.; HRMS (m/z): $[\text{Na}]^+$ calcd. for $\text{C}_{15}\text{H}_{30}\text{N}_4\text{O}_3$: 337.2210; found, 337.2209.

3.7.9 Preparation of S10

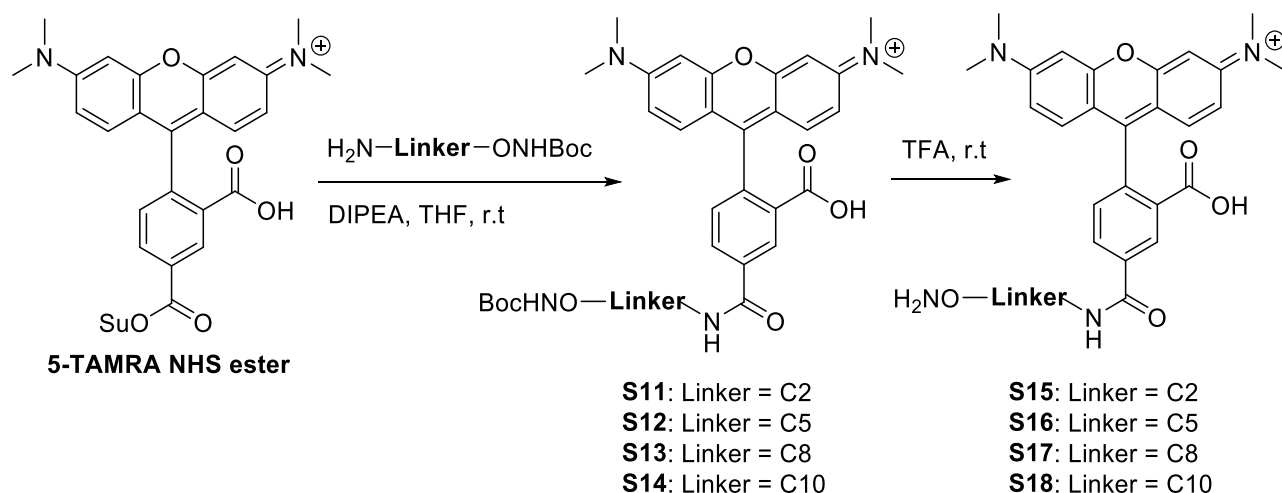


Supplementary Structure **S10**

Following the above general method for **S9** compound **S8** (221 mg, 0.7 mmol) gave **S10** (198.5 mg, 0.69 mmol, 98%) as a colorless oil.

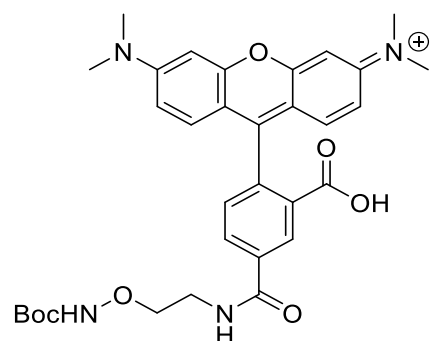
S10: TLC (DCM / MeOH / NH_4OH , v/v/v 4:1:0.5), R_f = 0.5. ^1H NMR (400 MHz MeOD): δ 3.74 (t, J = 6.6 Hz, 2H), 2.59 (t, J = 7.1 Hz, 2H), 1.60-1.53 (m, 2H), 1.40-1.48 (s, 11H), 1.36-1.29 (m, 12H).; ^{13}C NMR (100 MHz MeOD): δ 157.7, 80.4, 76.1, 48.5, 41.2, 32.4, 29.3, 29.3, 29.3, 29.2, 27.8, 27.3, 26.7, 25.7.; HRMS (m/z): $[\text{H}]^+$ calcd. for $\text{C}_{15}\text{H}_{32}\text{N}_2\text{O}_3$: 289.2486; found, 289.2489.

3.7.10 Synthesis of aminoxy-TAMRA probes



Scheme 2. Synthesis of aminoxy-TAMRA **1-4**.

3.7.11 Preparation of **S11**

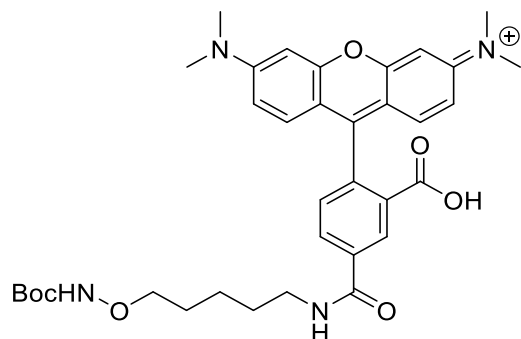


Supplementary Structure **S11**

Tert-butyl (2-aminoethoxy)carbamate (40 mg, 2 Eq, 0.19 mmol) was added to a mixture of 5-TAMRA-NHS ester (50 mg, 1 Eq, 95 μmol) and N,N-Diisopropylethylamine (61 mg, 83 μL , 5 Eq, 0.47 mmol) in THF (1 mL) and stirred for 8 hour at 25 °C. After the reaction was complete, reaction mixture was concentrated, extracted with DCM and water and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **S11** (51 mg, 87 μmol , 92 %) as a fuchsia solid.

S11: TLC (DCM / MeOH, v/v 10:1), R_f = 0.2. ^1H NMR (400 MHz CDCl_3): δ 8.59 (s, 1H), 8.55 (m, 1H), 8.24 (dd, J = 8.0, 1.6 Hz, 1H), 8.18 (br, 1H), 7.19 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 8.9 Hz, 2H), 6.42-6.40 (m, 4H), 3.97 (t, J = 4.8 Hz, 2H), 3.71 (q, J = 5.1 Hz, 2H), 3.00 (s, 12H), 1.46 (s, 9H).; ^{13}C NMR (100 MHz CDCl_3): δ 168.9, 166.2, 157.9, 153.9, 153.3, 136.1, 129.4, 109.9, 98.0, 82.2, 75.3, 42.4, 38.2, 29.7, 28.8.; HRMS (m/z): $[\text{H}]^+$ calcd. for $\text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_7$: 589.2657; found, 589.2659.

3.7.12 Preparation of S12

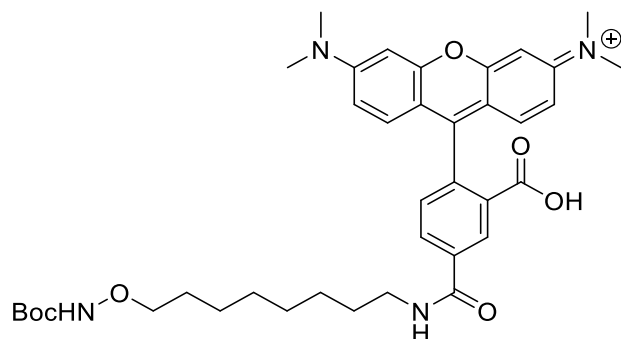


Supplementary Structure S12

Tert-butyl ((5-aminopentyl)oxy)carbamate (41.5 mg, 2 Eq, 0.19 mmol) was added to a mixture of 5-TAMRA-NHS ester (50 mg, 1 Eq, 95 μ mol) and N,N-Diisopropylethylamine (61 mg, 83 μ L, 5 Eq, 0.47 mmol) in THF (1 mL) and stirred for 8 hour at 25 $^{\circ}$ C. After the reaction was complete, reaction mixture was concentrated, extracted with DCM and water and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **S12** (57.98 mg, 91.92 μ mol, 97 %) as a fuchsia solid.

S12: TLC (DCM / MeOH, v/v 10:1), R_f = 0.2. ^1H NMR (400 MHz CDCl_3): δ 8.45 (s, 1H), 8.19 (d, J = 8.0 Hz, 1H), 7.67 (br, 1H), 7.16 (d, J = 8.0 Hz, 1H), 6.68 (s, 1H), 6.66 (s, 1H), 6.50 (s, 2H), 6.44 (d, J = 2.5 Hz, 1H), 6.42 (d, J = 2.5 Hz, 1H), 3.83 (t, J = 6.1 Hz, 2H), 3.45 (dd, J = 13.0, 6.6 Hz, 2H), 3.02 (s, 12H), 1.66-1.60 (m, 4H), 1.49-1.47 (m, 2H), 1.42 (s, 9H).; ^{13}C NMR (100 MHz CDCl_3): δ 168.9, 166.3, 157.0, 154.3, 136.5, 133.0, 129.6, 125.8, 125.3, 110.2, 108.5, 97.7, 81.2, 76.3, 40.4, 39.8, 28.8, 28.2, 27.3, 23.1.; HRMS (m/z): $[\text{H}]^+$ calcd. for $\text{C}_{35}\text{H}_{42}\text{N}_4\text{O}_7$: 631.3126; found, 631.3125.

3.7.13 Preparation of S13

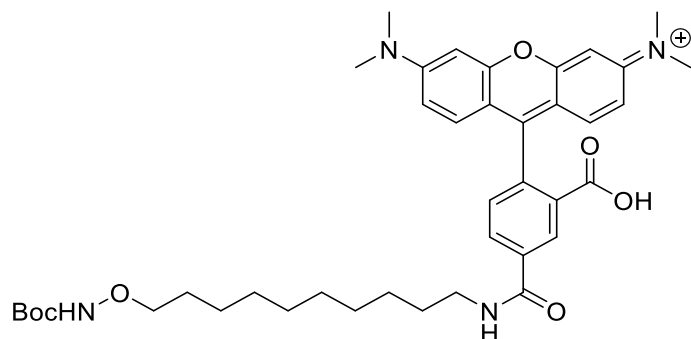


Supplementary Structure S13

Tert-butyl ((8-amino-octyl)oxy)carbamate (37 mg, 0.28 mL, 0.5 molar, 1.5 Eq, 0.14 mmol) was added to a mixture of 5-TAMRA-NHS ester (50 mg, 1 Eq, 95 μ mol) and N-ethyl-N-isopropylpropan-2-amine (37 mg, 50 μ L, 3 Eq, 0.28 mmol) in THF (1 mL) and stirred for 8 h at room temperature. After the reaction was complete, reaction mixture was concentrated, extracted with DCM and water and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **S13** (57 mg, 85 μ mol, 90 %) as a fuchsia solid.

S13: TLC (DCM / MeOH, v/v 10:1), R_f = 0.2. ^1H NMR (400 MHz CDCl_3): δ 8.42 (s, 1H), 8.16 (d, J = 7.9 Hz, 1H), 7.45-7.41 (m, 2H), 7.16 (d, J = 7.9 Hz, 1H), 6.70 (d, J = 9.0 Hz, 2H), 6.50 (d, J = 2.4 Hz, 2H), 6.44 (dd, J = 9.1, 2.5 Hz, 2H), 3.81 (t, J = 6.6 Hz, 2H), 3.42 (q, J = 6.7 Hz, 2H), 3.02 (s, 12H), 1.58 (m, 4H), 1.44 (s, 9H), 1.39-1.23 (m, 8H); ^{13}C NMR (100 MHz CDCl_3): δ 169.3, 166.1, 157.0, 153.4, 136.5, 134.1, 129.0, 125.0, 109.28, 106.9, 98.2, 81.4, 76.8, 40.3, 40.3, 29.7, 29.4, 29.2, 29.1, 28.2, 28.0, 26.8, 26.8, 25.8; HRMS (m/z): $[\text{H}]^+$ calcd. for $\text{C}_{38}\text{H}_{48}\text{N}_4\text{O}_7$: 673.3596; found, 673.3599.

3.7.14 Preparation of S14

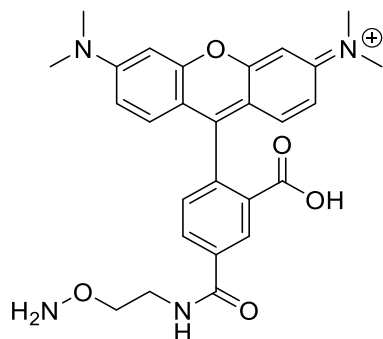


Supplementary Structure **S14**

Tert-butyl ((10-aminodecyl)oxy)carbamate (55 mg, 2 Eq, 0.19 mmol) was added to a mixture of 5-TAMRA-NHS ester (50 mg, 1 Eq, 95 μmol) and N-ethyl-N-isopropylpropan-2-amine (61 mg, 83 μL , 5 Eq, 0.47 mmol) in THF (1 mL) and stirred for 8 h at room temperature. After the reaction was complete, reaction mixture was concentrated, extracted with DCM and water and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **S14** (59 mg, 84 μmol , 89 %) as a fuchsia solid.

S14: TLC (DCM / MeOH, v/v 10:1), R_f = 0.2. ^1H NMR (400 MHz CDCl_3): δ 8.39 (s, 1H), 8.15 (d, J = 8.1 Hz, 1H), 7.35 (s, 1H), 7.14 (d, J = 7.9 Hz, 1H), 6.63 (d, J = 9.0 Hz, 2H), 6.48 (d, J = 2.5 Hz, 2H), 6.39 (dd, J = 9.2, 2.5 Hz, 2H), 3.80 (t, J = 6.7 Hz, 2H), 3.42 (q, J = 6.8 Hz, 2H), 2.99 (s, 12H), 1.57 (m, 4H), 1.44 (s, 9H), 1.33-1.23 (m, 12H); ^{13}C NMR (100 MHz CDCl_3): δ 168.8, 166.3, 156.9, 154.4, 153.7, 136.6, 133.1, 129.6, 126.0, 125.1, 110.3, 108.6, 97.6, 81.4, 77.3, 40.4, 40.3, 29.5, 29.48, 29.43, 29.3, 28.2, 28.0, 27.0, 25.8; HRMS (m/z): $[\text{H}]^+$ calcd. for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_7$: 701.3909; found, 701.3908.

3.7.15 Preparation of aminooxy-TAMRA **1**

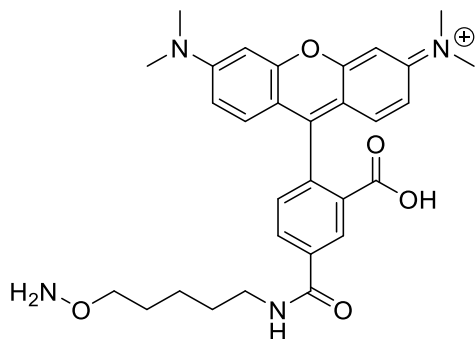


Structure 1: aminooxy-TAMRA **1**

TFA (1 mL) was added to **S11** (51 mg, 1 Eq, 87 μ mol) and stirred for 1 h at room temperature. After the reaction was complete, reaction mixture was concentrated and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **1** (29 mg, 59 μ mol, 68%) as a fuchsia solid.

1: TLC (DCM / MeOH v/v 10:1), R_f = 0.5. ^1H NMR (400 MHz MeOD): δ 8.57 (d, J = 1.9 Hz, 1H), 8.07 (dd, J = 7.8, 1.7 Hz, 1H), 7.35 (d, J = 7.9 Hz, 1H), 7.17 (s, 1H), 7.15 (s, 1H), 6.97 (d, J = 2.4 Hz, 1H), 6.94 (d, J = 2.4 Hz, 1H), 6.84 (s, 1H), 6.83 (s, 1H), 3.82 (t, J = 5.2 Hz, 2H), 3.65 (t, J = 5.2 Hz, 2H), 3.22 (s, 12H).; ^{13}C NMR (100 MHz MeOD): δ 168.4, 161.0, 158.2, 157.9, 136.4, 131.7, 130.5, 129.4, 114.4, 114.0, 96.7, 74.1, 40.2, 39.0.; HRMS (m/z): $[\text{H}]^+$ calcd. for $\text{C}_{27}\text{H}_{28}\text{N}_4\text{O}_5$: 489.2132; found, 489.2134.

3.7.16 Preparation of aminooxy-TAMRA **2**



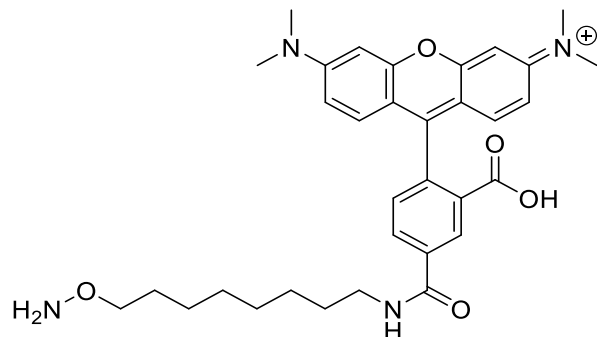
Structure 2: aminooxy-TAMRA **2**

Following the above general method for **1**, compound **S12** (57.98 mg, 1 Eq, 91.92 μ mol) gave **2** (42.18 mg, 79.49 μ mol, 86.47 %)

2: TLC (DCM / MeOH v/v 10:1), R_f = 0.5. ^1H NMR (400 MHz MeOD): δ 8.64 (s, 1H), 8.15 (d, J = 7.9 Hz, 1H), 7.40 (d, J = 7.9 Hz, 1H), 7.14 (s, 1H), 7.11 (s, 1H), 6.97 (d, J = 2.3 Hz, 1H), 6.95 (d, J = 2.3 Hz, 1H), 6.81 (s, 1H), 6.80 (s, 1H), 3.82 (t, J = 6.3 Hz, 2H), 3.44 (t, J = 7.0 Hz, 2H), 3.21 (s, 12H), 1.70-1.65 (m, 4H), 1.51-1.43 (m, 2H).; ^{13}C NMR (100 MHz MeOD): δ 167.8, 161.0, 158.1, 157.9, 136.6, 131.6, 130.7, 129.9, 114.6, 114.0, 96.7,

75.9, 40.3, 29.5, 28.1, 23.7.; HRMS (m/z): [H]⁺ calcd. for C₃₀H₃₄N₄O₅: 531.2602; found, 531.2602.

3.7.17 Preparation of aminooxy-TAMRA **3**

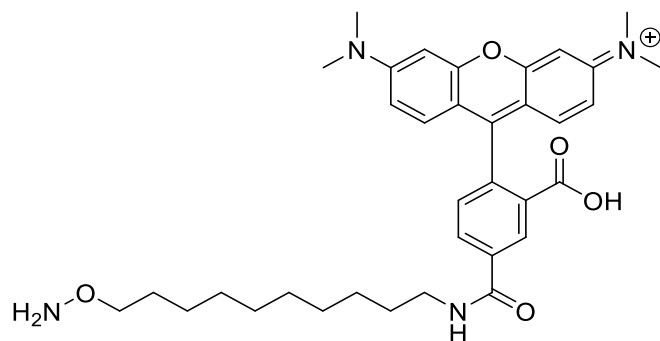


Structure 3: aminooxy-TAMRA **3**

Following the above general method for **1**, compound **S13** (57 mg, 1 Eq, 85 μmol) gave **3** (35 mg, 61 μmol, 72%)

3: TLC (DCM / MeOH v/v 10:1), R_f = 0.5; ¹H NMR (400 MHz, MeOD): δ 8.61 (s, 1H), 8.13 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.39 (d, *J* = 7.9 Hz, 1H), 7.14 (d, *J* = 9.5 Hz, 2H), 6.97 (dd, *J* = 9.5, 2.5 Hz, 2H), 6.83 (d, *J* = 2.4 Hz, 2H), 3.94 (t, *J* = 6.6 Hz, 1H), 3.76 (t, *J* = 6.6 Hz, 1H), 3.42 (t, *J* = 7.1 Hz, 2H), 3.22 (s, 12H), 1.67-1.56 (m, 4H), 1.42-1.34 (m, 9H); ¹³C NMR (100 MHz, MeOD): δ 167.8, 160.9, 158.2, 157.9, 155.7, 136.7, 136.5, 131.6, 130.6, 129.8, 114.5, 114.0, 96.7, 73.5, 40.5, 40.3, 29.8, 29.79, 29.76, 29.6, 29.4, 28.5, 27.4, 27.3, 26.4, 26.2; HRMS (m/z): [M+H]⁺ calcd. for C₃₃H₄₁N₄O₅: 573.3071; found, 573.3073.

3.7.18 Preparation of aminooxy-TAMRA **4**

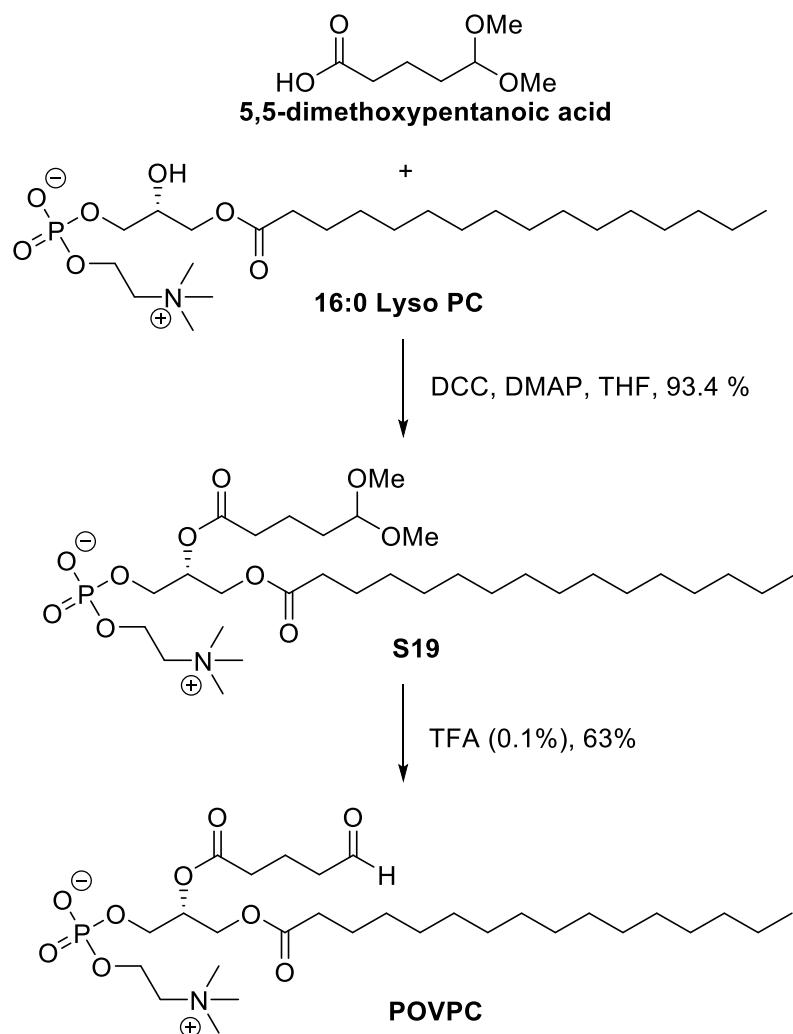


Structure 4: aminooxy-TAMRA **4**

Following the above general method for **1**, compound **S14** (59 mg, 1 Eq, 84 μmol) gave **4** (43.9 mg, 73.1 μmol, 87%)

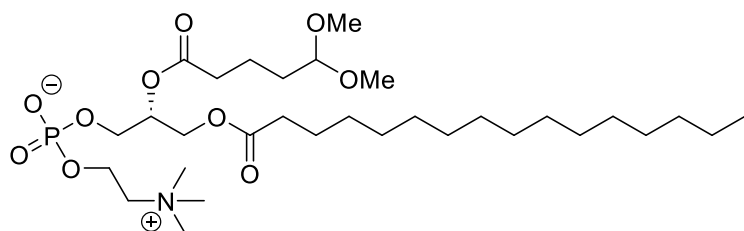
4: TLC (DCM / MeOH v/v 10:1), R_f = 0.5; ¹H NMR (400 MHz, MeOD): δ 8.61 (s, 1H), 8.11 (d, *J* = 7.9 Hz, 1H), 7.38 (d, *J* = 7.9 Hz, 1H), 7.14 (d, *J* = 9.3 Hz, 2H), 6.96 (dd, *J* = 9.5, 2.5 Hz, 2H), 6.83 (d, *J* = 2.5 Hz, 2H), 3.73 (t, *J* = 6.5 Hz, 2H), 3.42 (t, *J* = 7.2 Hz, 2H), 3.22 (s, 12H), 1.66-1.53 (m, 4H), 1.40-1.31 (m, 14H); ¹³C NMR (100 MHz, MeOD): δ 167.9, 161.2, 158.1, 157.3, 136.6, 136.4, 131.7, 130.6, 129.6, 114.5, 114.0, 96.7, 40.5, 40.3, 29.98, 29.96, 29.85, 29.81, 28.6, 27.4, 26.3; HRMS (m/z): [M+H]⁺ calcd. for C₃₅H₄₅N₄O₅: 601.3384; found, 601.3381.

3.7.19 Synthesis of POVPC



Scheme 3. Synthesis of **POVPC**

3.7.20 Preparation of S19



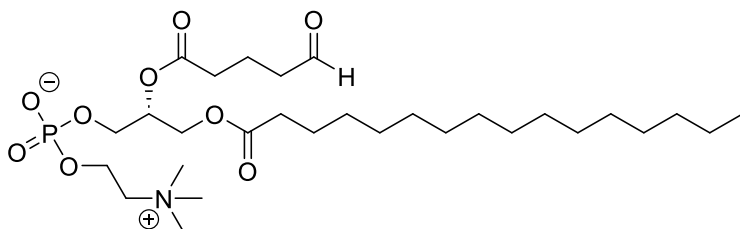
Supplementary Structure **S19**

Dicyclohexylmethanediimine (125 mg, 3 Eq, 605 μ mol) and N,N-dimethylpyridin-4-amine (73.9 mg, 3 Eq, 605 μ mol) were added to a mixture of **16:0 Lyso PC** (100 mg, 1 Eq, 202 μ mol) and 5,5-dimethoxypentanoic acid (98.2 mg, 3 Eq, 605 μ mol) in DCM (5 mL) and stirred for 24 hour at 25 °C. After

the reaction was complete, reaction mixture was purified by column chromatography (DCM / MeOH = 10:1, silica gel) to provide compound **S19** (120.63 mg, 188.54 μ mol, 93.4%) as a white sticky solid.

S19: TLC (DCM / MeOH / H₂O 60:25:4), R_f = 0.5. ¹H NMR (400 MHz CDCl₃): δ 5.14-5.11 (m, 1H), 4.32-4.24 (m, 4H), 4.07-4.02 (m, 1H), 3.89-3.86 (m, 2H), 3.79-3.75 (m, 2H), 3.32 (s, 9H), 3.24 (s, 3H), 2.27 (td, J = 6.9, 2.6 Hz, 2H), 2.21 (td, J = 7.7, 2.7 Hz, 2H), 1.59-1.48 (m, 7H), 1.19 (m, 27H), 0.81 (t, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 173.5, 172.7, 104.1, 70.5, 66.1, 62.8, 59.4, 54.2, 52.8, 34.0, 33.8, 31.9, 31.7, 29.7, 29.6, 29.6, 29.5, 29.3, 29.3, 29.1, 24.8, 22.6, 19.9, 14.1.; HRMS (m/z): [H]⁺ calcd. for C₃₁H₆₂NO₁₀P: 640.4184; found, 640.4186.

3.7.21 Preparation of POVPC

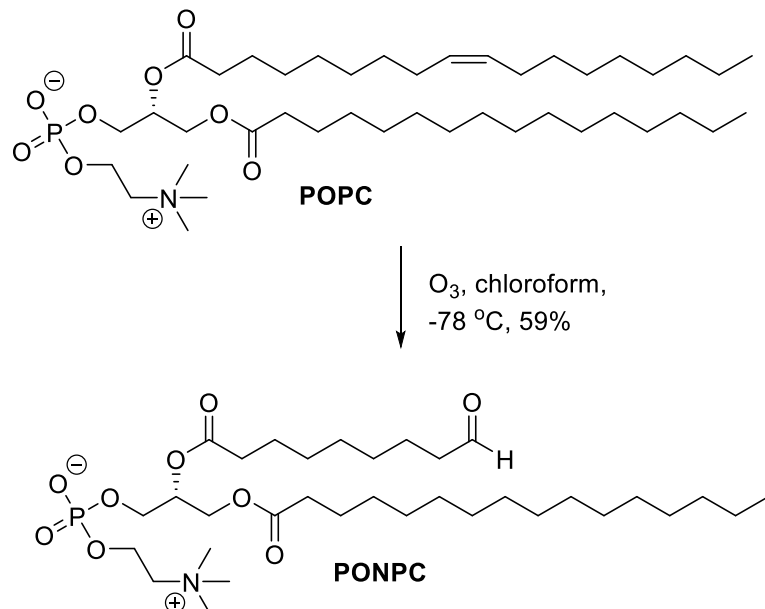


Structure 5: **POVPC**

Compound **S19** (16.91 mg, 1 Eq, 26.43 μ mol) was added H₂O, containing 0.1% v/v TFA, and the reaction was sonicated for 1 min, stirred at 25 °C for 9 h and lyophilized to yield the **POVPC** (14 mg, 24 μ mol, 89 %) as a white sticky solid. No flash column chromatography purification was performed.

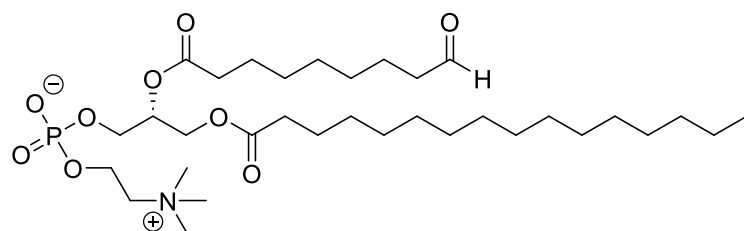
POVPC: TLC (DCM / MeOH / H₂O 60:25:4), R_f = 0.5; ¹H NMR (400 MHz, CDCl₃): δ 9.72 (t, J = 2.1 Hz, 1H), 5.16-5.13 (m, 1H), 4.33-4.25 (m, 3H), 4.11-4.05 (m, 1H), 3.90 (m, 2H), 3.79 (m, 2H), 3.33 (s, 9H), 2.53-2.49 (m, 2H), 2.35-2.20 (m, 4H), 1.89-1.84 (m, 2H), 1.53-1.50 (m, 2H), 1.22-1.1 (m, 27H), 0.84 (t, J = 7.1 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 201.9, 173.6, 172.4, 70.9, 66.2, 63.7, 62.8, 59.4, 54.3, 42.8, 34.1, 33.2, 32.0, 29.7, 29.7, 29.6, 29.4, 29.4, 29.2, 24.9, 22.7, 17.3, 14.2; HRMS (m/z): [M+H]⁺ calcd. for C₂₉H₅₇NO₉P: 594.3765; found, 594.3761.

3.7.22 Synthesis of PONPC



Scheme 4. Synthesis of **PONPC**

3.7.23 Preparation of PONPC

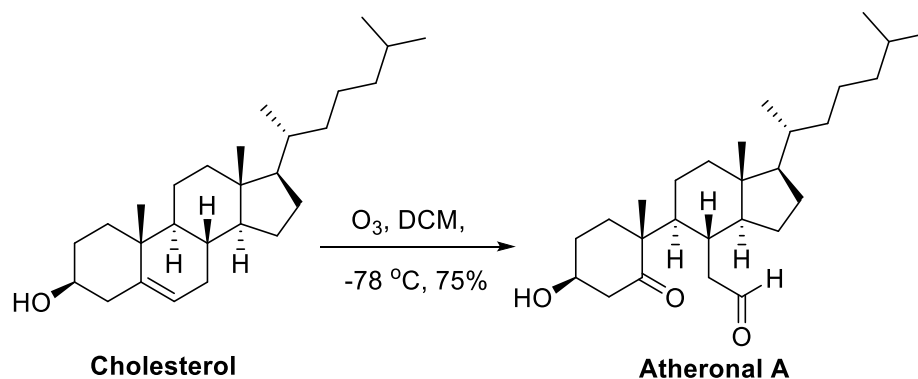


Structure 6: **PONPC**

A ~0.05 M solution of **POPC** (300 mg, 1 Eq, 395 μmol) in chloroform was cooled to -78 °C and then subjected to ozone for 5-15 minutes. Pure oxygen was then allowed to flow through the blue solution for 30 seconds. The dimethylsulfane (123 mg, 0.15 mL, 5 Eq, 1.97 mmol) was added to the clear colorless solution. The flask was allowed to warm to room temperature for 1 hour. The solvent was evaporated and purified by column chromatography (DCM / MeOH = 20:1, silica gel) to provide **PONPC** (150 mg, 231 μmol , 58.5 %) as a white sticky solid.

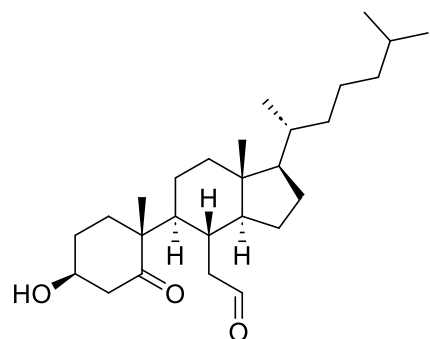
PONPC: TLC (DCM / MeOH / H₂O 60:25:4), R_f = 0.5; ¹H NMR (400 MHz, CDCl₃): δ 9.63 (t, J = 1.9 Hz, 1H), 5.09-5.05 (m, 1H), 4.28-4.17 (m, 3H), 4.01-3.96 (m, 1H), 3.84-3.79 (m, 2H), 3.69 (m, 2H), 3.25 (s, 9H), 2.30 (t, J = 7.3 Hz, 1H), 2.18-2.12 (m, 5H), 1.50-1.40 (m, 7H), 1.18-1.12 (d, J = 26.2 Hz, 33H), 0.74 (t, J = 6.7 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 202.8, 173.5, 173.0, 70.59, 70.51, 66.2, 63.3, 62.9, 59.3, 54.2, 43.8, 34.19, 34.11, 31.9, 29.70, 29.69, 29.66, 29.54, 29.53, 29.36, 29.34, 29.33, 29.18, 29.16, 29.0, 28.9, 28.8, 24.9, 24.8, 24.78, 24.72, 22.6, 21.9, 14.1; HRMS (m/z): [$M+H$]⁺ calcd. for C₃₃H₆₅NO₉P: 650.4391; found, 650.4392.

3.7.24 Synthesis of Atheronal A



Scheme 5. Synthesis of **Atheronal A**

3.7.25 Preparation of Atheronal A

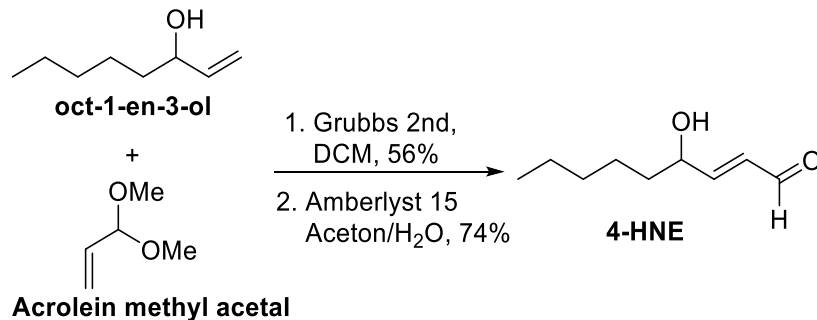


Structure 7. **Atheronal A**

A ~0.05 M solution of cholesterol (500 mg, 1 Eq, 1.29 mmol) in DCM (26 mL) was cooled to -78 °C and then subjected to ozone for 5-15 minutes. Pure oxygen was then allowed to flow through the blue solution for 30 seconds. The dimethylsulfane (402 mg, 0.48 mL, 5 Eq, 6.47 mmol) was added to the clear colorless solution. The flask was allowed to warm to room temperature for 1 hour. The solvent was evaporated and purified by column chromatography (EtOAc/Hex = 10:1, silica gel) to provide **Atheronal A** (400 mg, 955 μmol , 73.9 %) as a white foam.

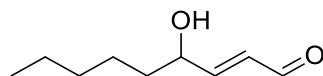
Atheronal A: TLC (Hex / EtOAc 1:1), R_f = 0.5; ^1H NMR (400 MHz, CDCl_3): δ 9.54 (d, J = 1.7 Hz, 1H), 4.39 (t, J = 3.5 Hz, 1H), 3.00 (dd, J = 13.6, 4.0 Hz, 1H), 2.33-2.23 (m, 2H), 2.20 (dd, J = 18.3, 3.4 Hz, 1H), 2.09 (td, J = 11.3, 4.0 Hz, 1H), 1.98 (dt, J = 12.5, 3.2 Hz, 1H), 1.88 – 1.54 (m, 8H), 1.50-1.39 (m, 2H), 1.38-1.10 (m, 9H), 1.10-0.87 (m, 10H), 0.84 (d, J = 6.5 Hz, 3H), 0.79 (dd, J = 6.7, 2.0 Hz, 7H), 0.61 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 218.1, 202.9, 70.9, 56.0, 54.4, 52.6, 46.8, 44.3, 42.5, 42.3, 39.8, 39.4, 35.9, 35.7, 34.6, 34.1, 28.0, 27.8, 27.6, 25.3, 23.7, 23.0, 22.8, 22.5, 18.5, 17.6, 11.5; HRMS (m/z): $[\text{M}+\text{Na}]^+$ calcd. for $\text{C}_{27}\text{H}_{46}\text{O}_3\text{Na}$: 441.3339; found, 441.3338.

3.7.26 Synthesis of 4-HNE



Scheme 6. Synthesis of **4-HNE**

3.7.27 Preparation of 4-HNE



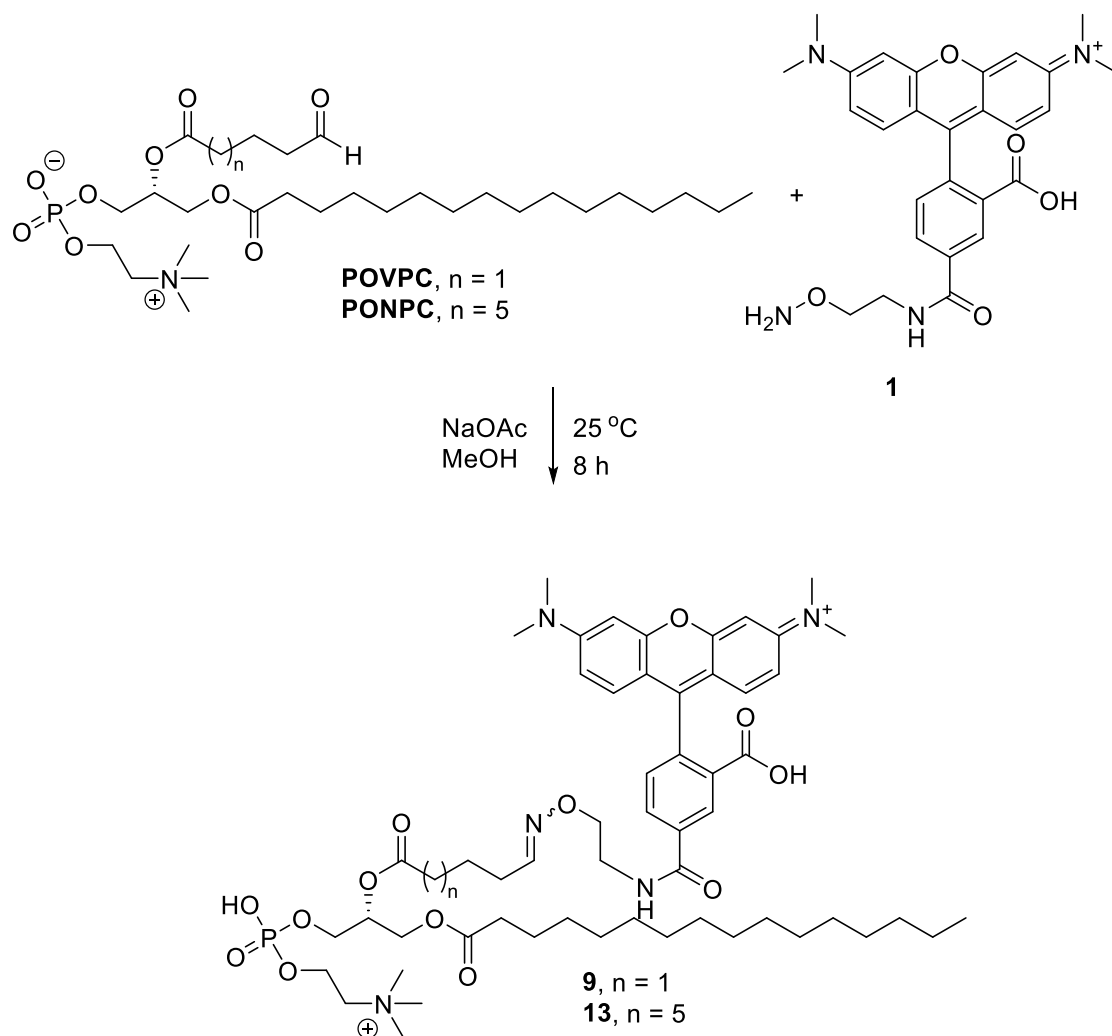
Structure 8: **4-HNE**

The Hoveyda-Grubbs 2nd catalyst (49.0 mg, 0.025 Eq, 78.0 μ mol) was added in two equal portion at $t = 0$ and $t = 5$ h to a solution of oct-1-en-3-ol (400 mg, 1 Eq, 3.12 mmol) and 3,3-dimethoxyprop-1-ene (956 mg, 3 Eq, 9.36 mmol) in dry, oxygen free DCM (10 mL). After stirring for further 20 h at room temperature, the solvent was evaporated and the residue was purified by flash column chromatography (Hex:EtOAc = 6:1, silica gel) to give the (E)-1,1-dimethoxynon-2-en-4-ol (350 mg, 1.73 mmol, 55.5 %) as a black oil.

Amberlyst 15 resin (35 mg, 1 Eq, 1.73 mmol) was added to a mixture of (E)-1,1-dimethoxynon-2-en-4-ol (350 mg, 1 Eq, 1.73 mmol) in acetone (3 mL) and water (0.5 mL) and stirred for 6 hour at 25 °C. After the reaction was complete, reaction mixture was purified by column chromatography (Hex / EtOAc = 6:1, silica gel) to provide product **4-HNE** (200 mg, 1.28 mmol, 74.0 %) as a colorless oil.

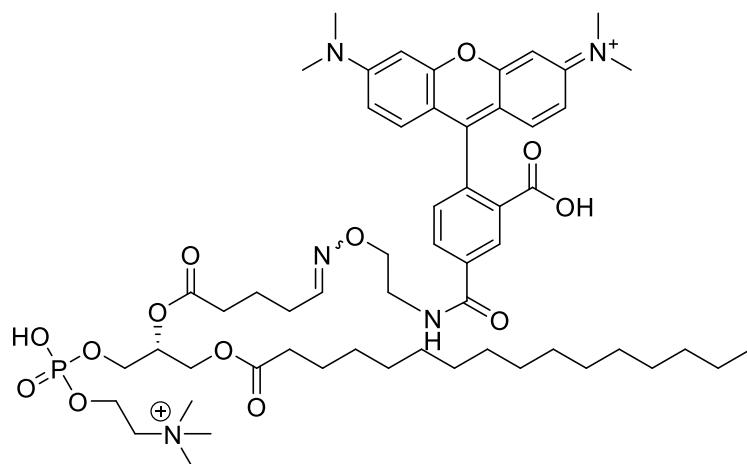
4-HNE: TLC (Hex / EtOAc 3:1), $R_f = 0.3$; 9.46 (d, $J = 8.0$ Hz, 1H), 6.80 (dd, $J = 15.6, 4.6$ Hz, 1H), 6.22 (ddd, $J = 15.6, 7.9, 1.6$ Hz, 1H), 4.36-4.31 (m, 1H), 3.19 (br, 1H), 1.57-1.51 (m, 2H), 1.39-1.18 (m, 8H), 0.86 – 0.78 (m, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ 194.3, 160.3, 130.4, 70.9, 36.3, 31.6, 24.9, 22.5, 14.0; HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_9\text{H}_{17}\text{O}_2$: 157.1223; found, 157.1222.

3.7.28 Synthesis of aldehyde phospholipid standards **9** and **13**



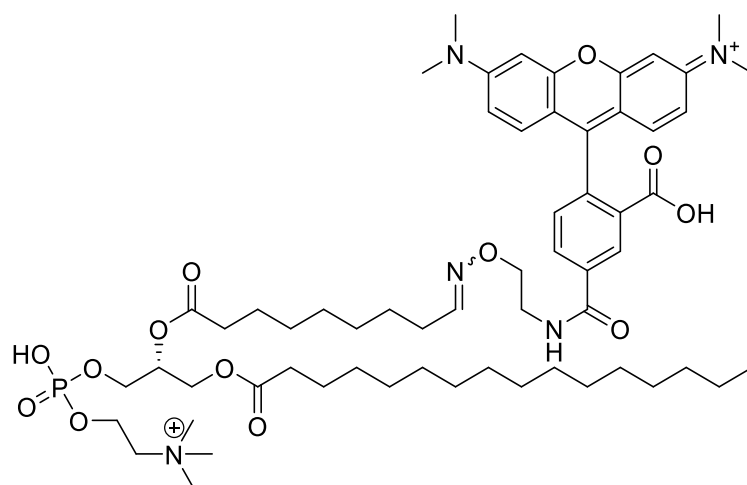
Scheme 7. Synthesis of **9** and **13**

3.7.29 Preparation of POVPC-oxime standard **9**



Structure 9: POVPC-oxime standard

3.7.30 Preparation of PONPC-oxime standard **13**



Structure 13: PONPC-oxime standard

Following the above general method for 9, PONPC (20 mg, 1 Eq, 31 μmol) gave 13 (14.25 mg, 12.7 μmol , 41%) as a fuchsia solid.

13: TLC (DCM / MeOH / H₂O 60:25:4), R_f = 0.4; ¹H NMR (400 MHz, CDCl₃) δ 8.45 (m, 1H), 8.24-8.22 (m, 1H), 7.45-7.42 (m, 1H), 7.30 (m, 2H), 6.72-6.69 (m, 2H), 6.57-6.51 (m, 2H), 5.17 (m, 1H), 4.31-3.66 (m, 8H), 3.27 (s, 12H), 3.06 (s, 9H), 2.30-2.21 (m, 4H), 1.56-1.47 (m, 6H), 1.66-1.28 (m, 30H), 0.85 (t, J = 6.1 Hz, 3H).; ¹³C NMR (100 MHz, CDCl₃) δ 176.3, 173.6, 173.4, 173.2, 166.0, 153.1, 152.2, 138.1, 136.4, 129.5, 128.5, 127.9, 98.0, 77.3, 73.5, 72.0, 71.6, 70.9, 70.6, 66.3, 64.3, 63.8, 63.7, 62.8, 59.4, 59.4, 54.4, 40.6, 34.4, 34.2, 34.2, 34.1, 32.0, 29.8, 29.6, 29.4, 29.2, 28.87, 28.80, 28.3, 28.28, 28.21, 26.5, 24.9, 24.8, 24.6, 24.4, 22.82, 14.2; HRMS (m/z): [M]⁺ calcd. for C₆₀H₉₁N₅O₁₃P, 1120.6346; found, 1120.6348.

3.8 Supplementary tables and figures

3.8.1 Determination of LogP values for aminoxy-TAMRA probes

Table 3.1 LogP values determined by LC–MS shake-flask method.

LogP values were determined using the shake-flask method with mutually saturated n-octanol and water, followed by LC–MS quantitation

Compound	logP
1	-1.95
2	-1.38
3	0.279
4	0.886

3.8.2 Chemoselectivity experiments

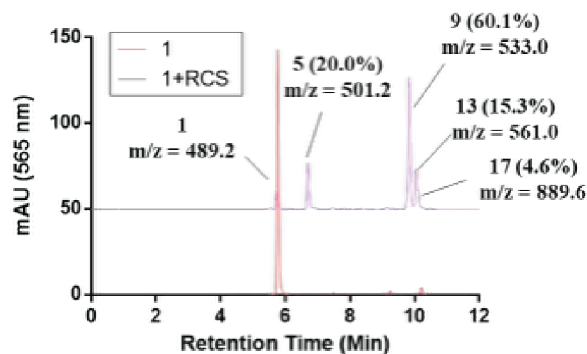
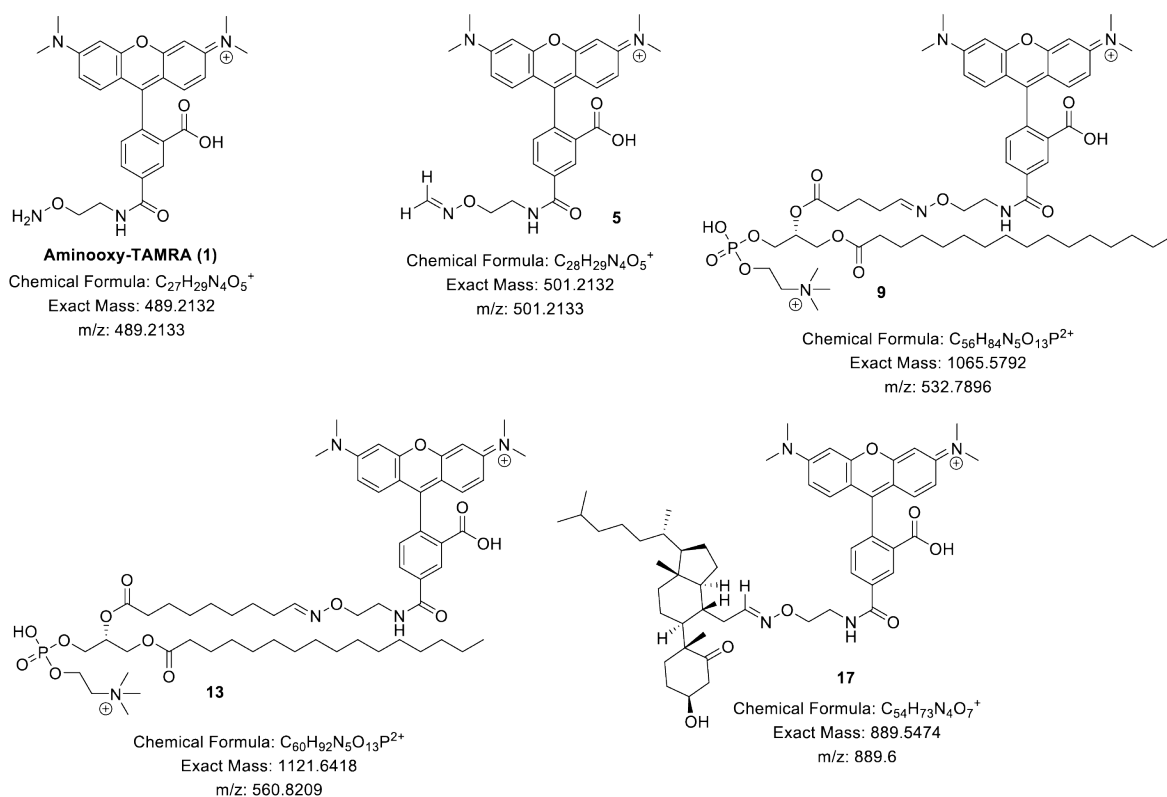


Figure S3.13. Chemoselectivity of Aminoxy-TAMRA (1).

(A) Chemical structure of detected compounds in LC-MS. (B) HPLC-DAD (565 nm) traces of 100 mM of Aminoxy-TAMRA (1) in PBS buffer (pH 7.4) before (bottom) and after (top) reacting with a panel of RCS (100 mM). Summing the two phospholipid aldehyde oxime peaks (m/z 533.0 and 561.0; 60.1% and 15.3%) and comparing to the formaldehyde oxime (m/z 501.2; 20.0%) and atheronal A oxime (m/z 889.6; 4.6%) give an 75.4% preference for phospholipid aldehydes.

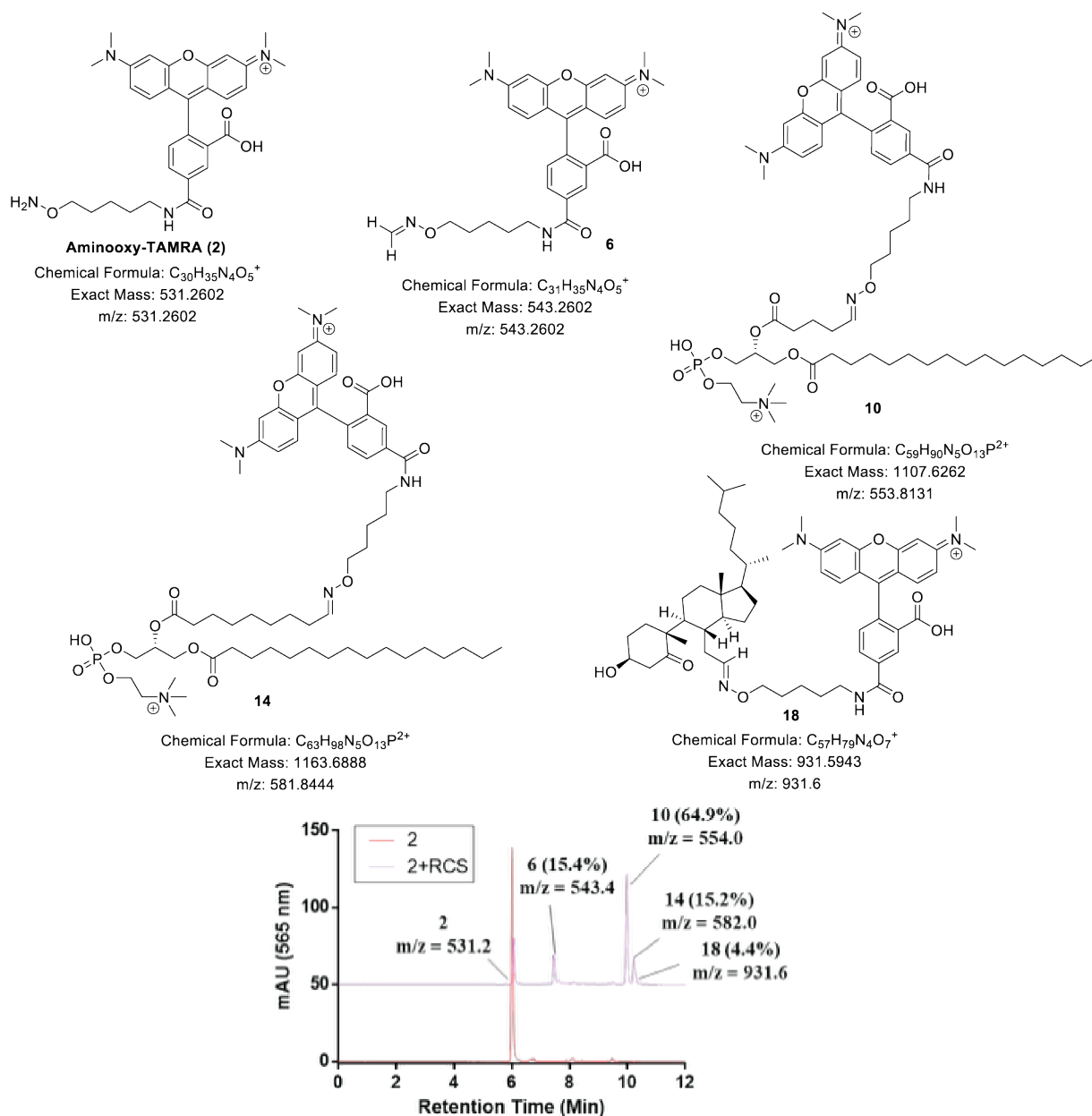


Figure S3.14. Chemoselectivity of Aminoxy-TAMRA (2).

(A) Chemical structure of detected compounds in LC-MS. (B) HPLC-DAD (565 nm) traces of 100 mM of Aminoxy-TAMRA (2) in PBS buffer (pH 7.4) before (bottom) and after (top) reacting with a panel of RCS (100 mM). Summing the two phospholipid aldehyde oxime peaks (m/z 554.0 and 582.0; 64.9% and 15.2%) and comparing to the formaldehyde oxime (m/z 543.4; 15.4%) and atheronal A oxime (m/z 889.6; 4.4%) give an 80.1% preference for phospholipid aldehydes.

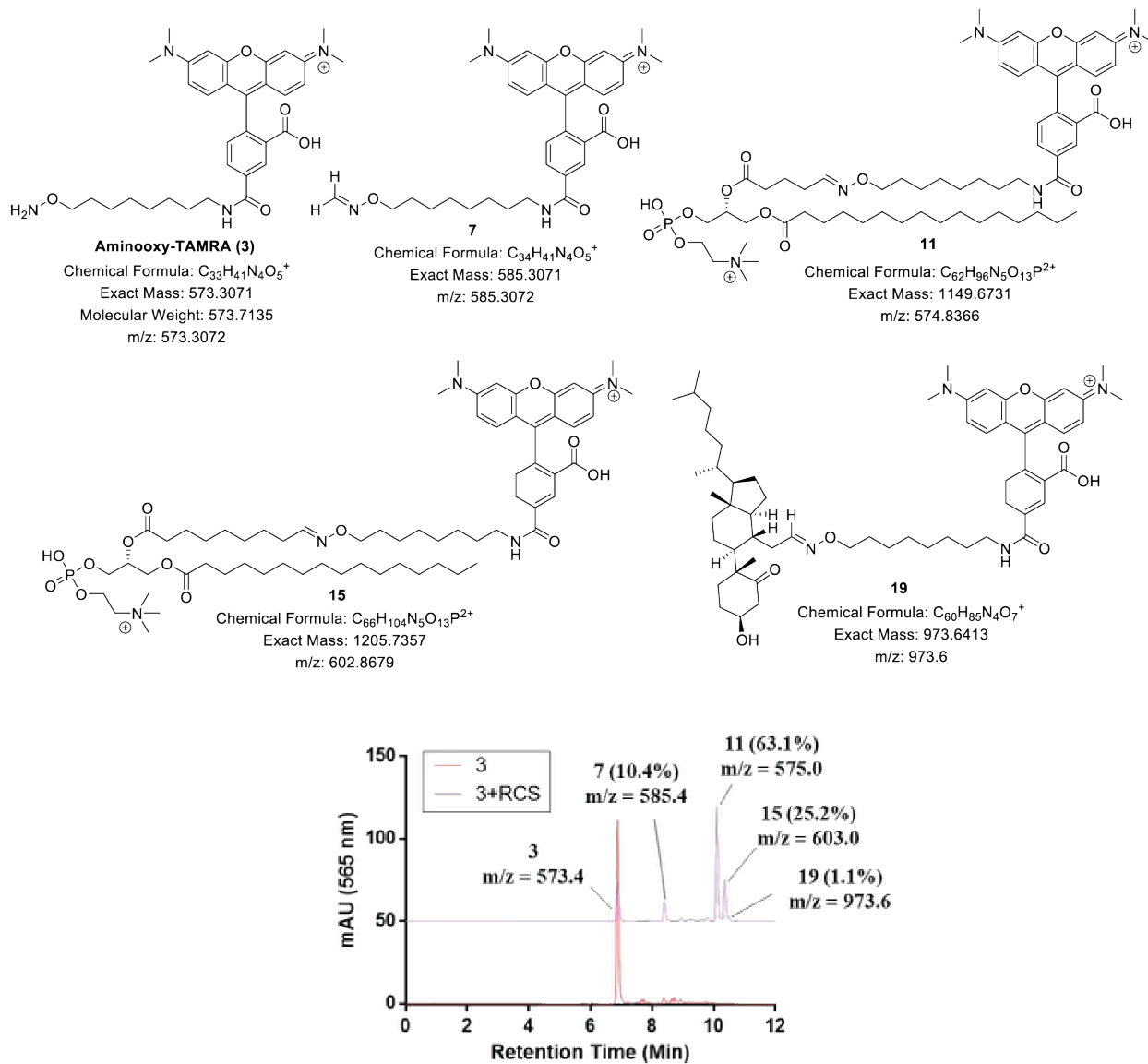


Figure S3.15. Chemoselectivity of Aminoxy-TAMRA (3).

(A) Chemical structure of detected compounds in LC-MS. (B) HPLC-DAD (565 nm) traces of 100 mM of Aminoxy-TAMRA (3) in PBS buffer (pH 7.4) before (bottom) and after (top) reacting with a panel of RCS (100 mM). Summing the two phospholipid aldehyde oxime peaks (m/z 575.0 and 603.0; 63.1% and 25.2%) and comparing to the formaldehyde oxime (m/z 585.4; 10.4%) and atheronal A oxime (m/z 973.6; 1.1%) give an 88.3% preference for phospholipid aldehydes.

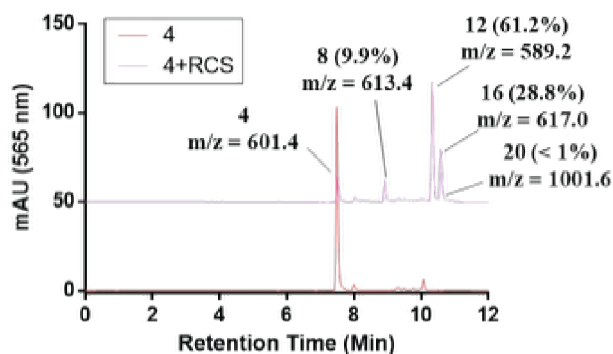
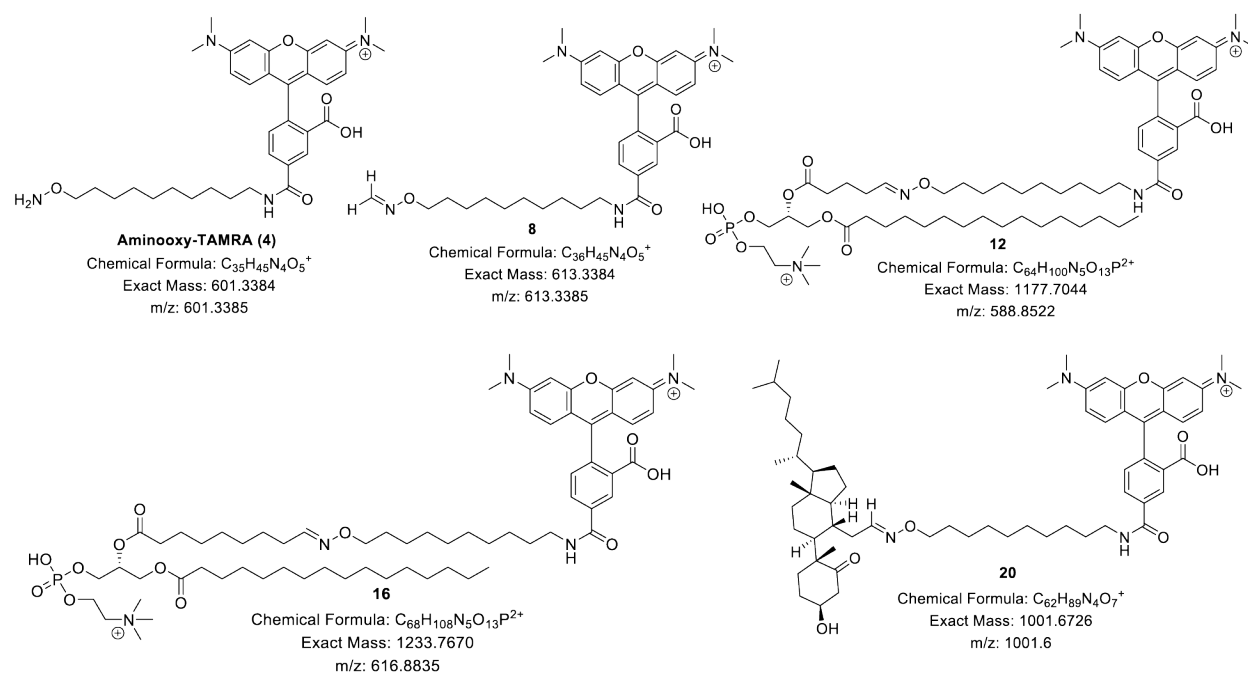


Figure S3.16. Chemoselectivity of Aminoxy-TAMRA (4).

(A) Chemical structure of detected compounds in LC-MS. (B) HPLC-DAD (565 nm) traces of 100 mM of Aminoxy-TAMRA (4) in PBS buffer (pH 7.4) before (bottom) and after (top) reacting with a panel of RCS (100 mM). Summing the two phospholipid aldehyde oxime peaks (m/z 589.2 and 617.0; 61.2% and 28.8%) and comparing to the formaldehyde oxime (m/z 613.4; 9.9%) and atheronal A oxime (m/z 1001.6; <1%) give an 90.0% preference for phospholipid aldehydes.

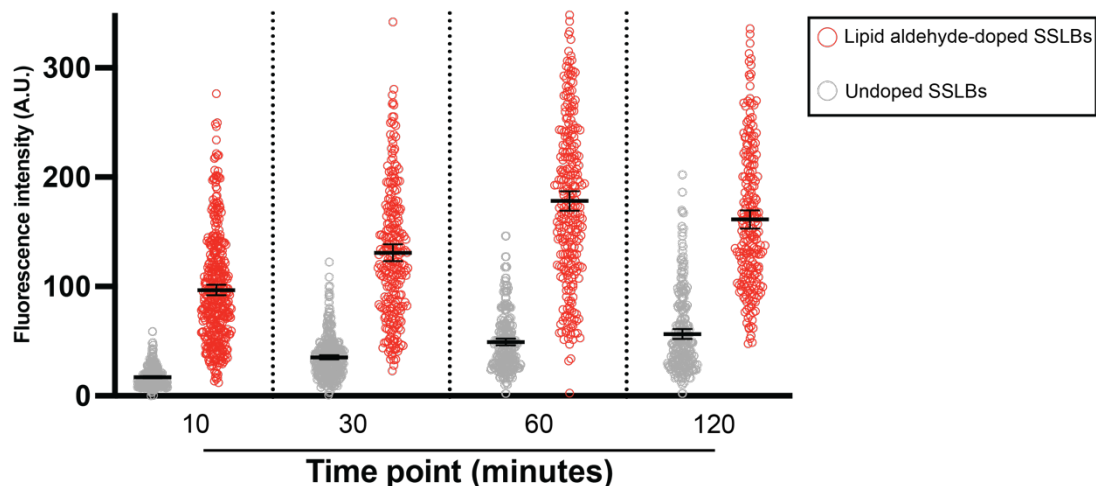


Figure S3.17. Time course of aminooxy-TAMRA-1 incorporation into SSLBs measured by fluorescence intensity.

Fluorescence intensities of SSLBs doped (red) or undoped (grey) with the phospholipid aldehyde 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC) and incubated with 1 μ M aminooxy-TAMRA 1 at pH 7.4 in 10 mM phosphate buffer before washing away unreacted dye and imaging at various time points (10, 30, 60, and 120 minutes). Data points represent the average fluorescence of individual SSLBs as calculated by mean pixel intensity. Black bars represent the median fluorescence, and error bars represent SEM, with $n = 500$ SSLBs measured per condition. Because SSLB samples undergo multiple wash steps, the plotted fluorescence reflects membrane-retained oxime adducts and unreacted aminooxy-TAMRA dye rather than total conversion.

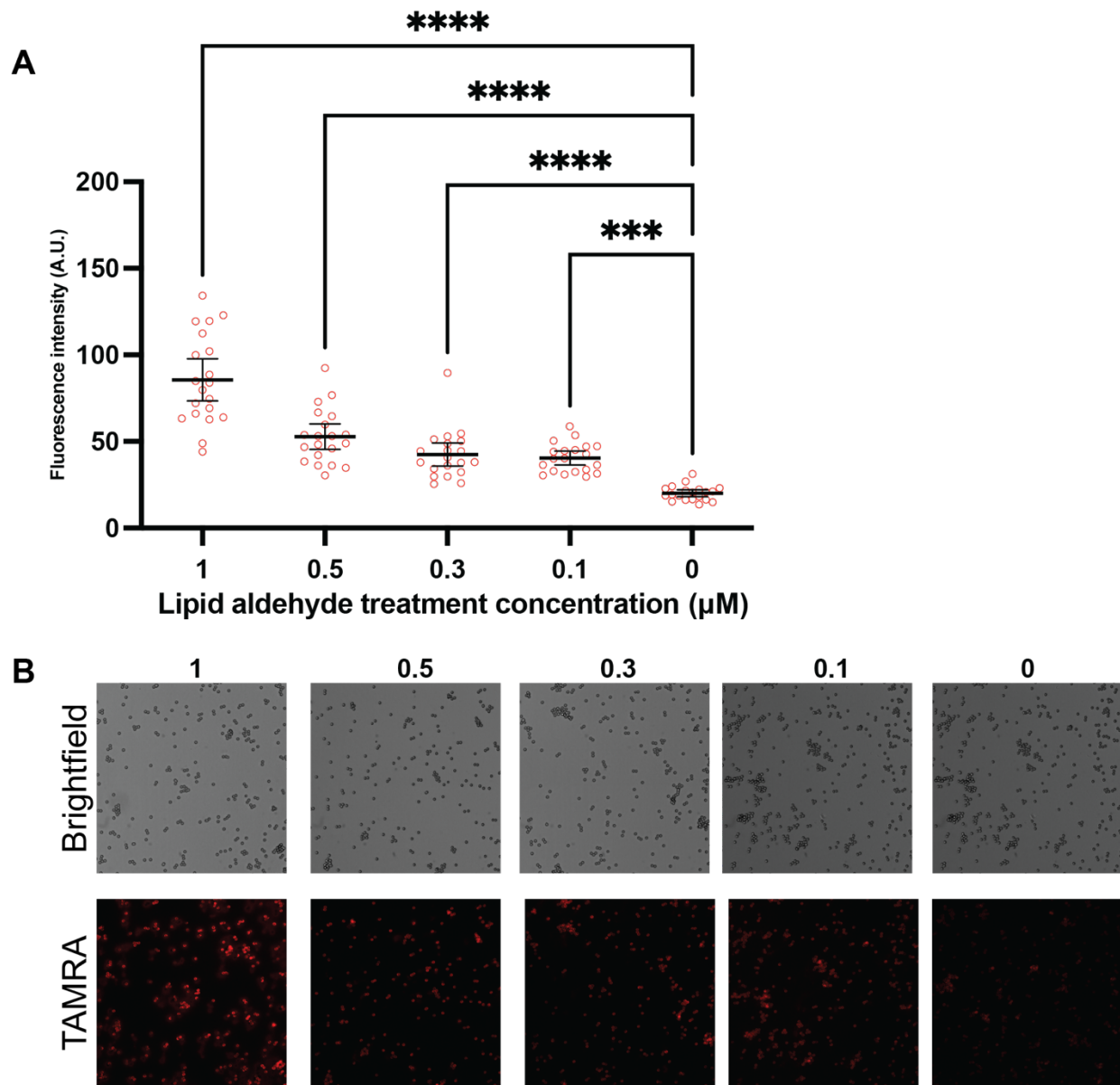


Figure S3.18. Limit of detection of lipid aldehyde doped SSLBs treated with aminooxy-TAMRA **1**.

Fluorescence intensities (**A**) and confocal micrographs (**B**) of SSLBs doped with several concentrations of the phospholipid aldehyde 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC) and incubated for 30 minutes with 1 μM aminooxy-TAMRA **1** at pH 7.4 in 10 mM phosphate buffer.

Fluorescence intensities were compared by one-way ANOVA with Dunnett's post-hoc test versus the undoped control (**** $P < 0.0001$), with $n = 20$ SSLBs measured per condition.

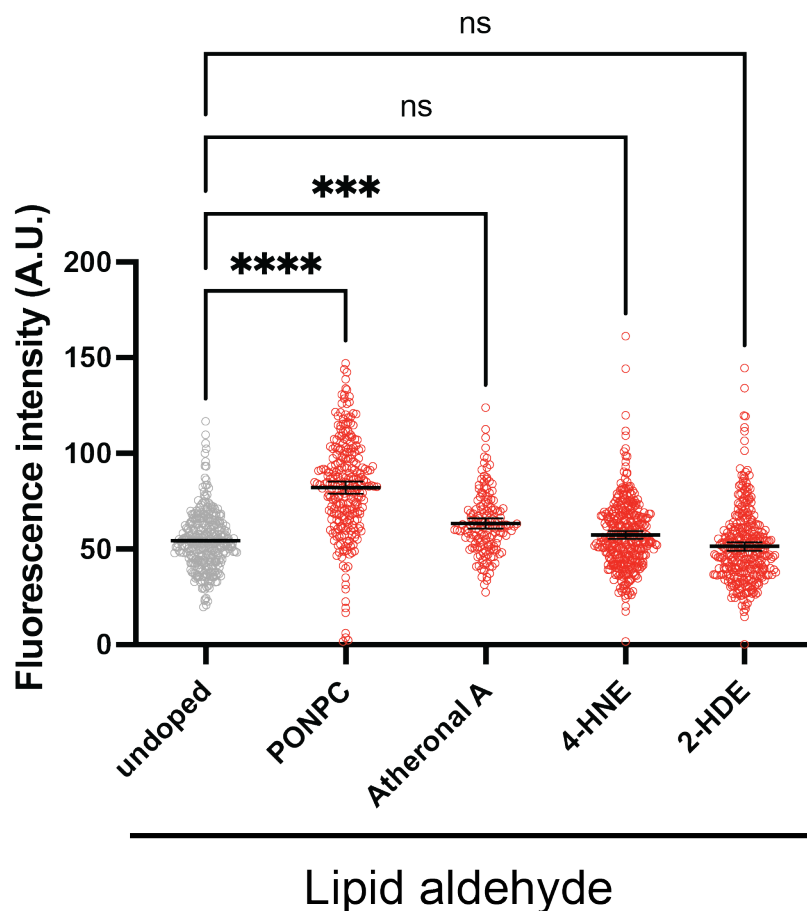


Figure S3.19. Fluorescence intensities of SSLBs doped with lipid aldehyde species of several classes.

1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC), atheronal A, 4-hydroxynonenal (4-HNE), 4-hydroxydecenal (4-HDE) incubated for 30 minutes with 1 μ M aminooxy-TAMRA 1 at pH 7.4 in 10 mM phosphate buffer. Fluorescence intensities were compared by one-way ANOVA with Dunnett's post-hoc test versus the undoped control (**** $P < 0.0001$; ns, not significant). Data points represent the average fluorescence of individual SSLBs as calculated by mean pixel intensity. Black bars represent the median fluorescence, and error bars represent SEM, with $n = 500$ SSLBs measured per condition. Because SSLB samples undergo multiple wash steps, the plotted fluorescence reflects membrane-retained oxime adducts and unreacted aminooxy-TAMRA dye rather than total conversion.

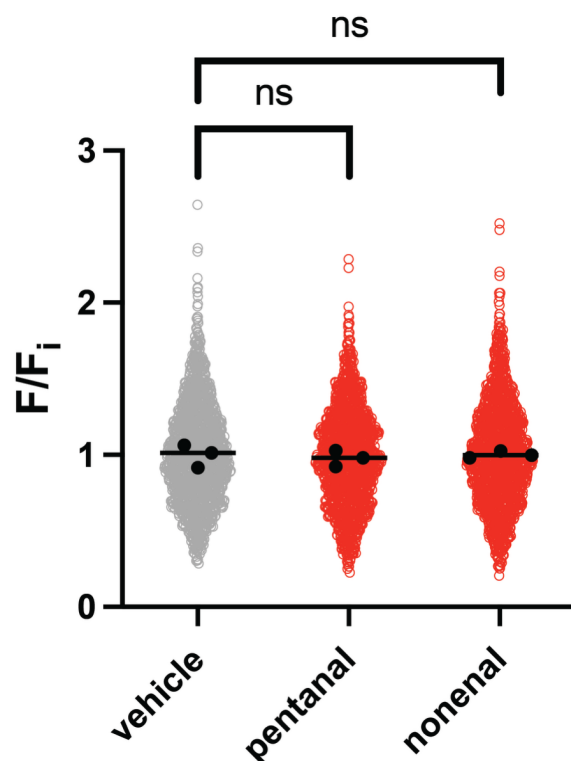


Figure S3.20. Fluorescence intensities of HT-1080 cells treated with alkyl aldehydes and aminooxy-TAMRA 1.

Fluorescence intensities of live HT-1080 fibrosarcoma cells treated first for 30 minutes with alkyl aldehydes pentanal and nonenal at a concentration of 20 μ M followed by washout and subsequent incubation for 30 minutes with 1 μ M aminooxy-TAMRA 1 at pH 7.4 in 10 mM phosphate buffer. Images were collected by automated confocal microscopy imaging and images were segmented and analyzed using CellProfiler. Cells from 10 images per well of a 96-well plate were averaged, with 3 wells measured per condition. The means of each well were averaged and compared between samples by one-way ANOVA with Dunnett's post-hoc test (ns, not significant).

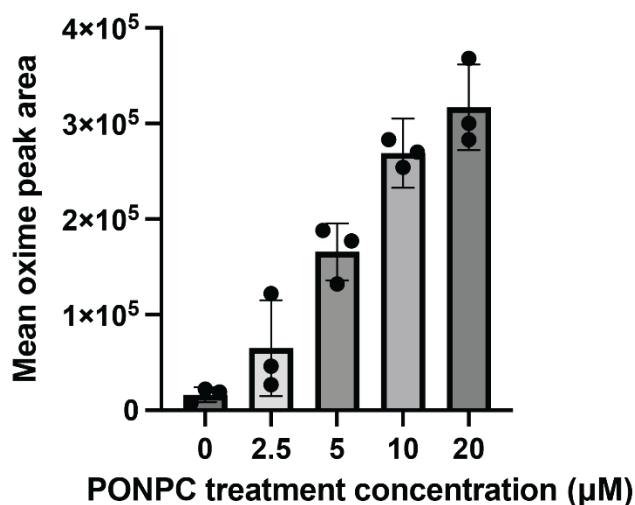


Figure S3.21. Mean peak areas of oxime adducts of PONPC detected in the lipid extracts of cells by LC/MS-MS.

Cells were treated first with PONPC (30 minutes, 20 μM) and stained with aminooxy-TAMRA 1 (30 minutes, 1 μM) before they were collected by scraping into ice cold phosphate-buffered saline. Lipids were extracted by the method of Bligh and Dyer and extracts were analyzed by LC/MS-MS. The identity of the TAMRA adduct of PONPC was confirmed by comparison to an authentic standard and quantified by integrating the peak area.

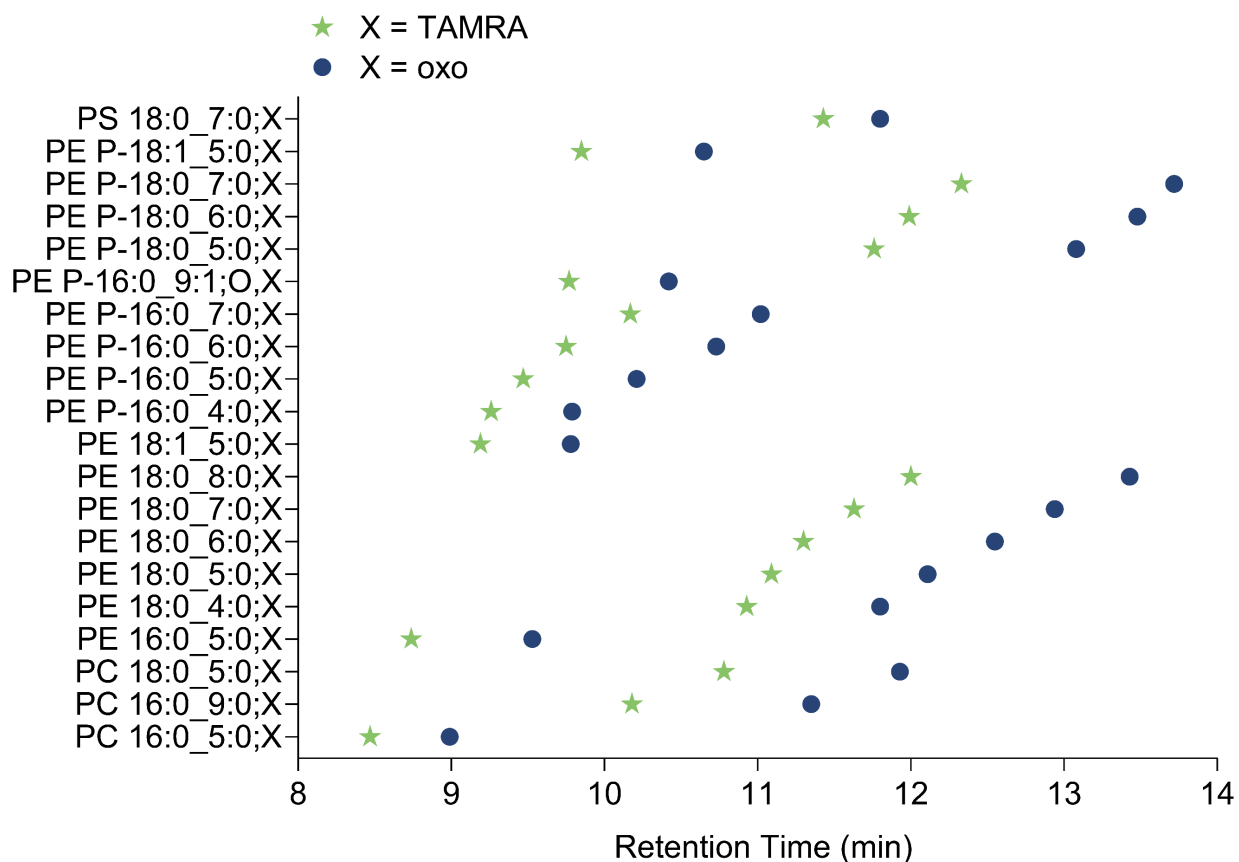


Figure S3.22. Comparison of retention times of parent oxidized phospholipids and TAMRA oxime derivatives.

TAMRA oxime derivatives (green stars) of oxidized lipids were analyzed by HRMS. TAMRA oxime derivatives consistently showed shorter retention times than parent oxidized lipids detected in the same extracts, providing additional validation of their authentic detection.

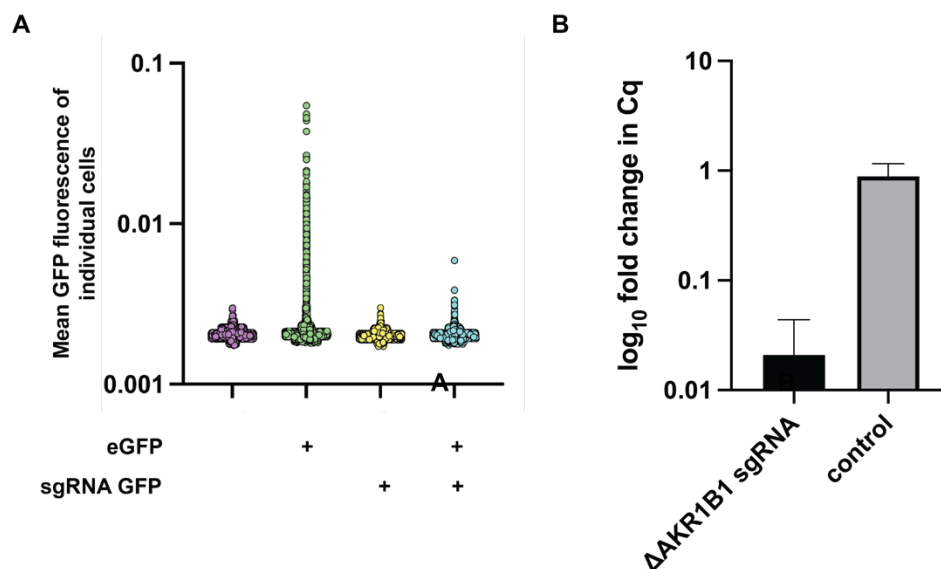
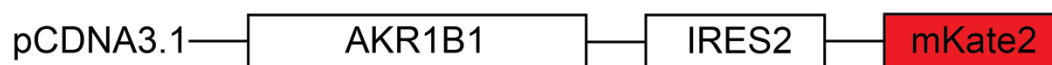


Figure S3.23. CRISPRi-mediated knockdown of AKR1B1 in HT-1080 cells.

(A) Co-transfection of HT-1080 dCas9 cells with a GFP-expressing lentiviral cassette with an sgRNA targeting GFP results in loss of GFP fluorescence. **(B)** Transfection of HT-1080 dCas9 cells with an sgRNA targeting AKR1B1 results in loss of the mRNA transcript from cell lysates as shown by qPCR analysis.

A



B

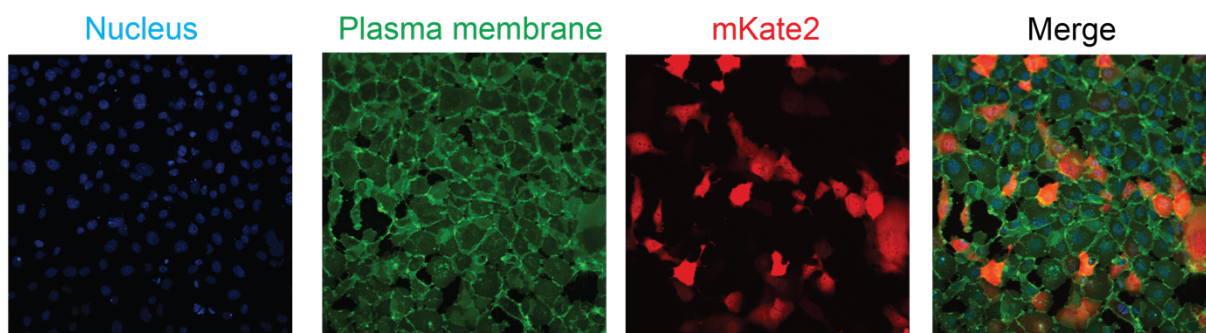


Figure S3.24. Overexpression of AKR1B1 in HT-1080 cells.

(A) Schematic of AKR1B1 construct with mKate2 reporter. **(B)** Confocal micrographs showing expression of the mKate2 reporter in transiently transfected cells, demonstrating expression of the construct.

Table S3.2. Confirmed phospholipid oxime targets detected in pooled lipid extracts subjected to quantification and analysis in subsequent experiments.

Compound	Neutral Formula	Adduct	m/z	z	RT (min)
PC 16:0_5:0;T	C ₅₆ H ₈₂ O ₁₃ N ₅ P	[M+2H]	532.7896	2	8.5
PC 16:0_9:0;T	C ₆₀ H ₉₀ O ₁₃ N ₅ P	[M+2H]	560.8209	2	10.2
PC 18:0_5:0;T	C ₅₈ H ₈₆ O ₁₃ N ₅ P	[M+2H]	546.8053	2	10.8
PE 16:0_5:0;T	C ₅₃ H ₇₆ O ₁₃ N ₅ P	[M+2H]	511.7661	2	8.7
PE 18:0_4:0;T	C ₅₄ H ₇₈ O ₁₃ N ₅ P	[M+2H]	518.7740	2	10.9
PE 18:0_5:0;T	C ₅₅ H ₈₀ O ₁₃ N ₅ P	[M+2H]	525.7818	2	11.1
PE 18:0_6:0;T	C ₅₆ H ₈₂ O ₁₃ N ₅ P	[M+2H]	532.7896	2	11.3
PE 18:0_7:0;T	C ₅₇ H ₈₄ O ₁₃ N ₅ P	[M+2H]	539.7974	2	11.6
PE 18:0_8:0;T	C ₅₈ H ₈₆ O ₁₃ N ₅ P	[M+2H]	546.8053	2	12.0
PE 18:1_5:0;T	C ₅₅ H ₇₈ O ₁₃ N ₅ P	[M+2H]	524.7740	2	9.2
PE P-16:0_4:0;T	C ₅₂ H ₇₄ O ₁₂ N ₅ P	[M+2H]	496.7609	2	9.3
PE P-16:0_5:0;T	C ₅₃ H ₇₆ O ₁₂ N ₅ P	[M+2H]	503.7687	2	9.5
PE P-16:0_6:0;T	C ₅₄ H ₇₈ O ₁₂ N ₅ P	[M+2H]	510.7765	2	9.8
PE P-16:0_7:0;T	C ₅₅ H ₈₀ O ₁₂ N ₅ P	[M+2H]	517.7843	2	10.2
PE P-16:0_9:1;O,T	C ₅₇ H ₈₂ O ₁₃ N ₅ P	[M+2H]	538.7896	2	9.8
PE P-18:0_5:0;T	C ₅₅ H ₈₀ O ₁₂ N ₅ P	[M+2H]	517.7843	2	11.8
PE P-18:0_6:0;T	C ₅₆ H ₈₂ O ₁₂ N ₅ P	[M+2H]	524.7922	2	12.0
PE P-18:0_7:0;T	C ₅₇ H ₈₄ O ₁₂ N ₅ P	[M+2H]	531.8000	2	12.3
PE P-18:1_5:0;T	C ₅₅ H ₇₈ O ₁₂ N ₅ P	[M+2H]	516.7765	2	9.9
PS 18:0_7:0;T	C ₅₈ H ₈₄ O ₁₅ N ₅ P	[M+2H]	561.7924	2	11.4

3.9 Chapter Appendix: Spectra of synthesized compounds

3.9.1 NMR spectra of compound S3

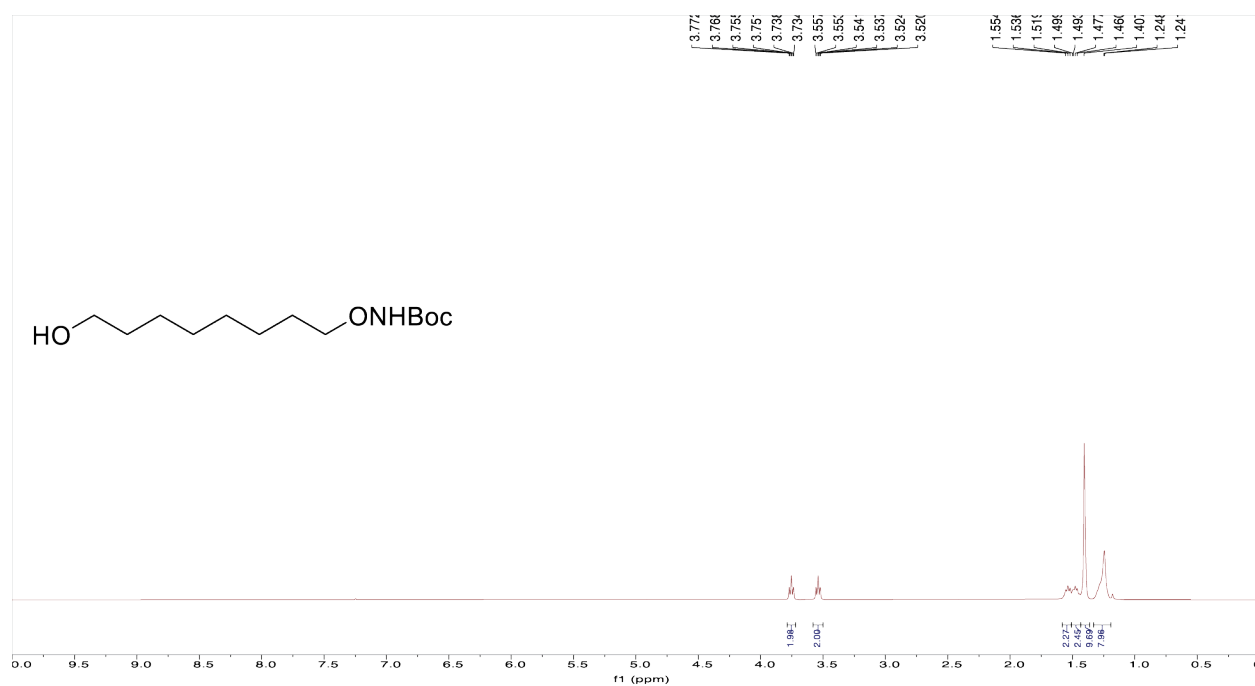


Figure 3.25. ¹H NMR Spectrum of compound S3 (400 MHz, CDCl₃)

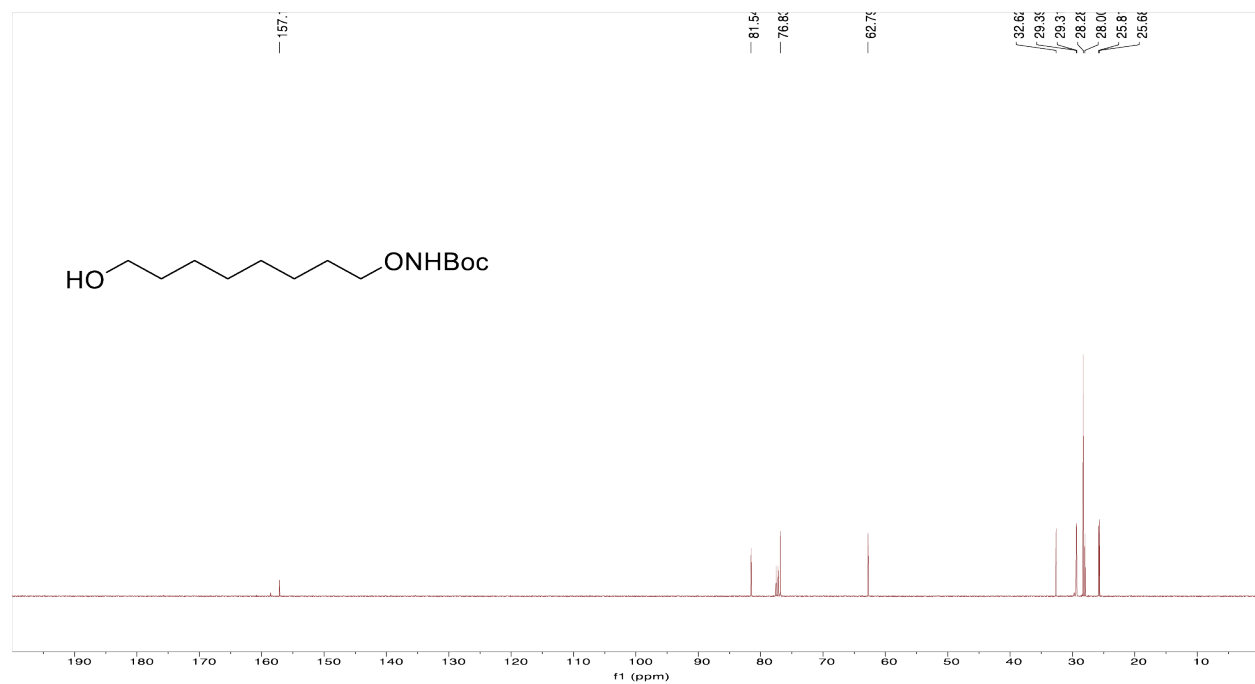


Figure S3.26. ¹³C NMR Spectrum of compound S3 (100 MHz, CDCl₃)

3.9.2 NMR spectra of compound S5

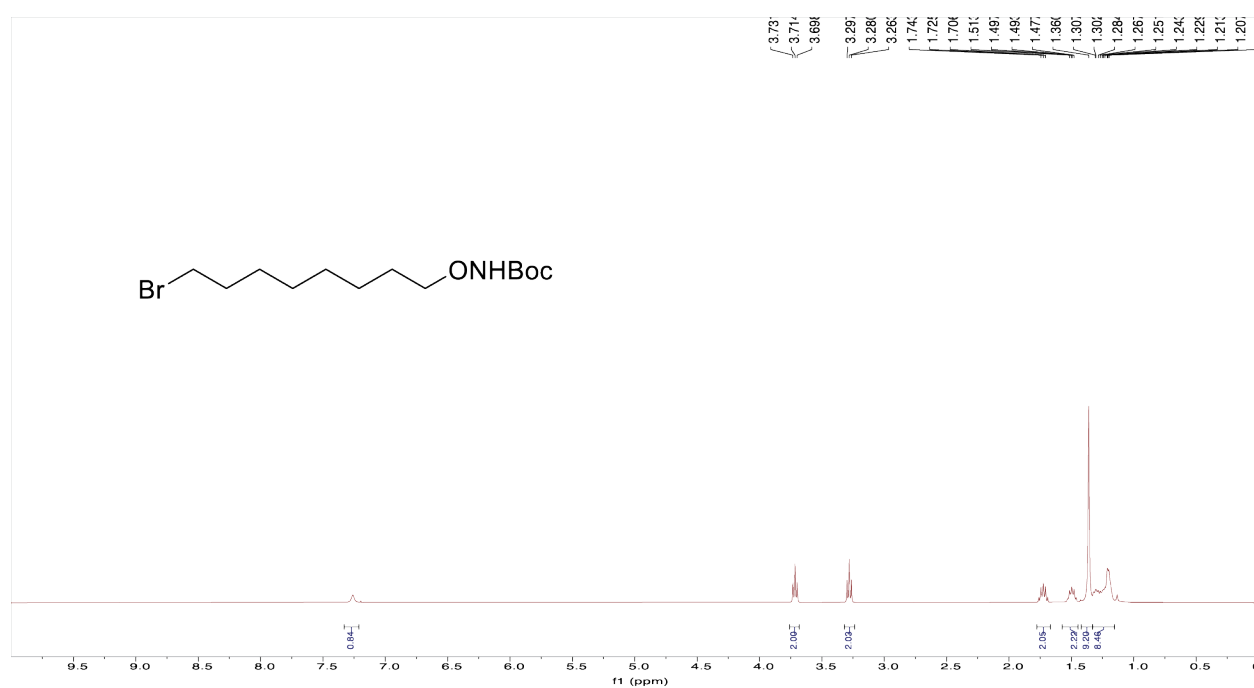


Figure S3.27. ^1H NMR Spectrum of compound S5 (400 MHz, CDCl_3)

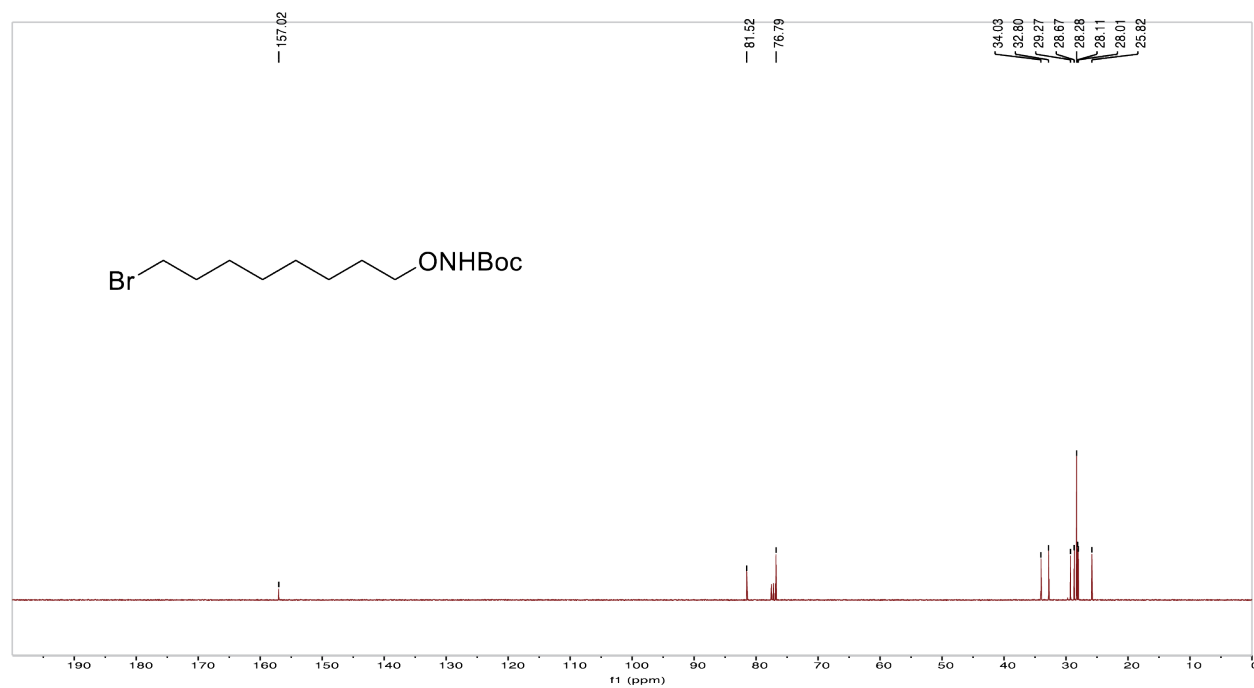


Figure S3.28. ^{13}C NMR Spectrum of compound S5 (100 MHz, CDCl_3)

3.9.3 NMR Spectra of compound S7

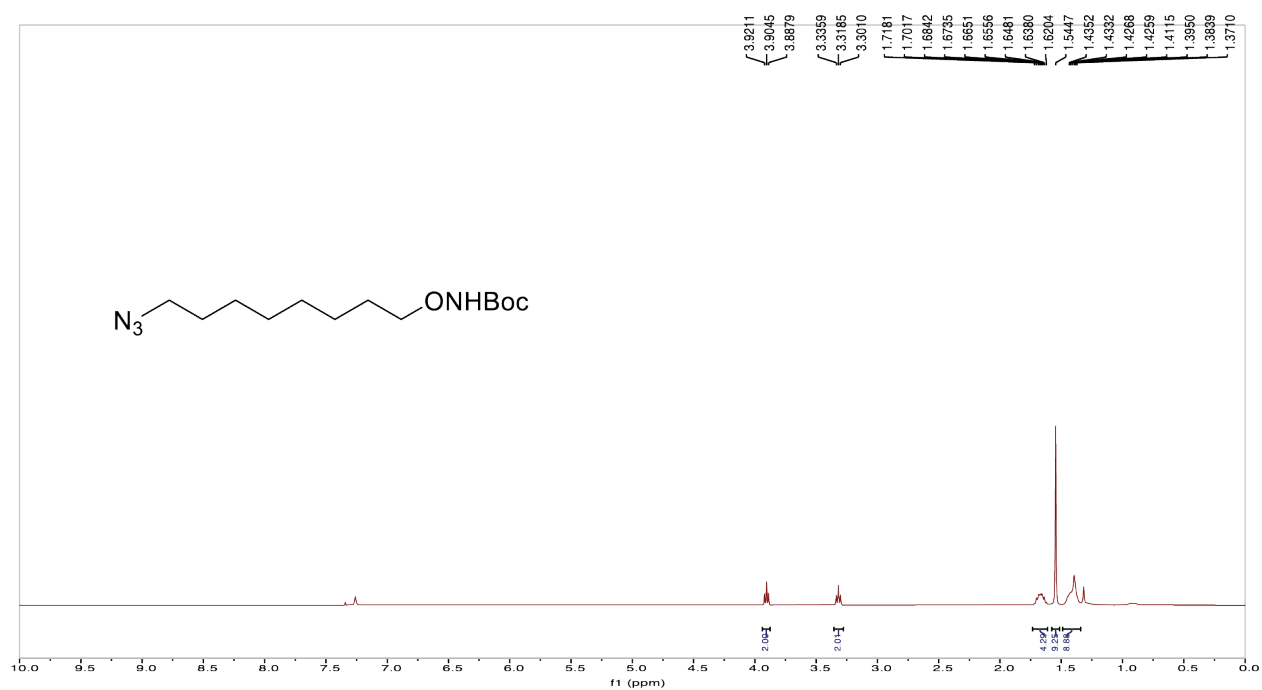


Figure S3.29. ¹H NMR Spectrum of compound S7 (400 MHz, CDCl₃)

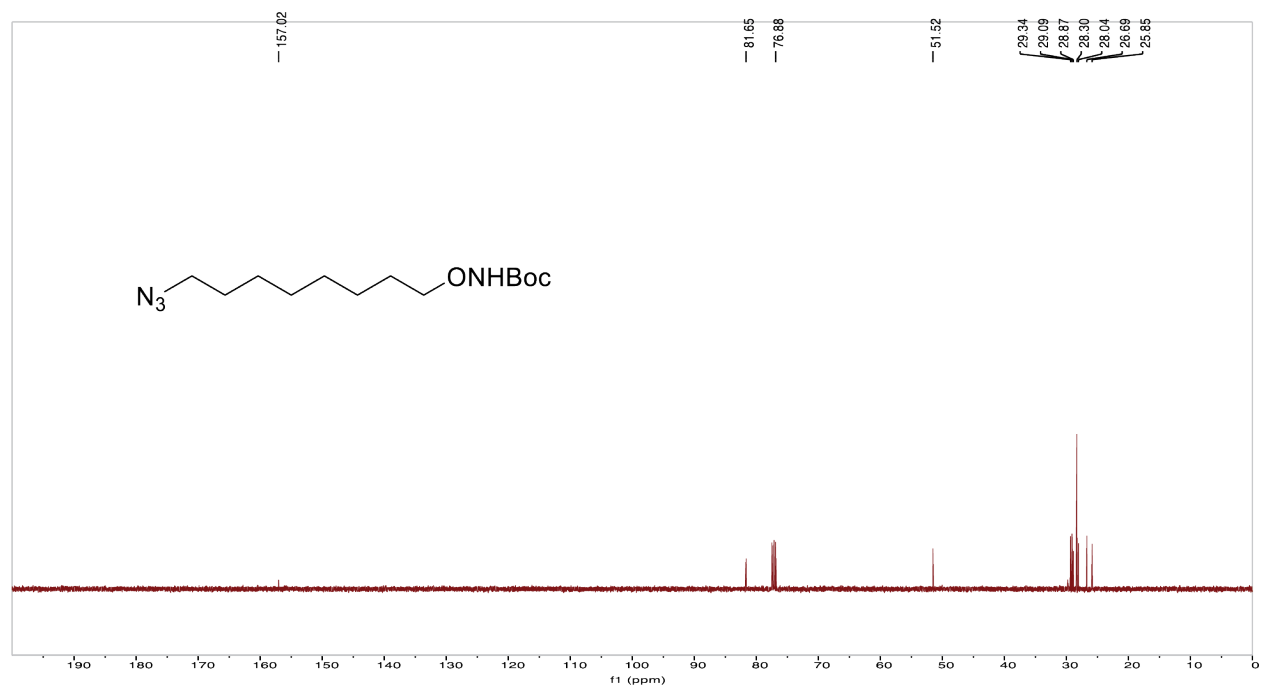


Figure S3.30. ¹³C NMR Spectrum of compound S7 (100 MHz, CDCl₃)

3.9.4 NMR spectra of compound S9

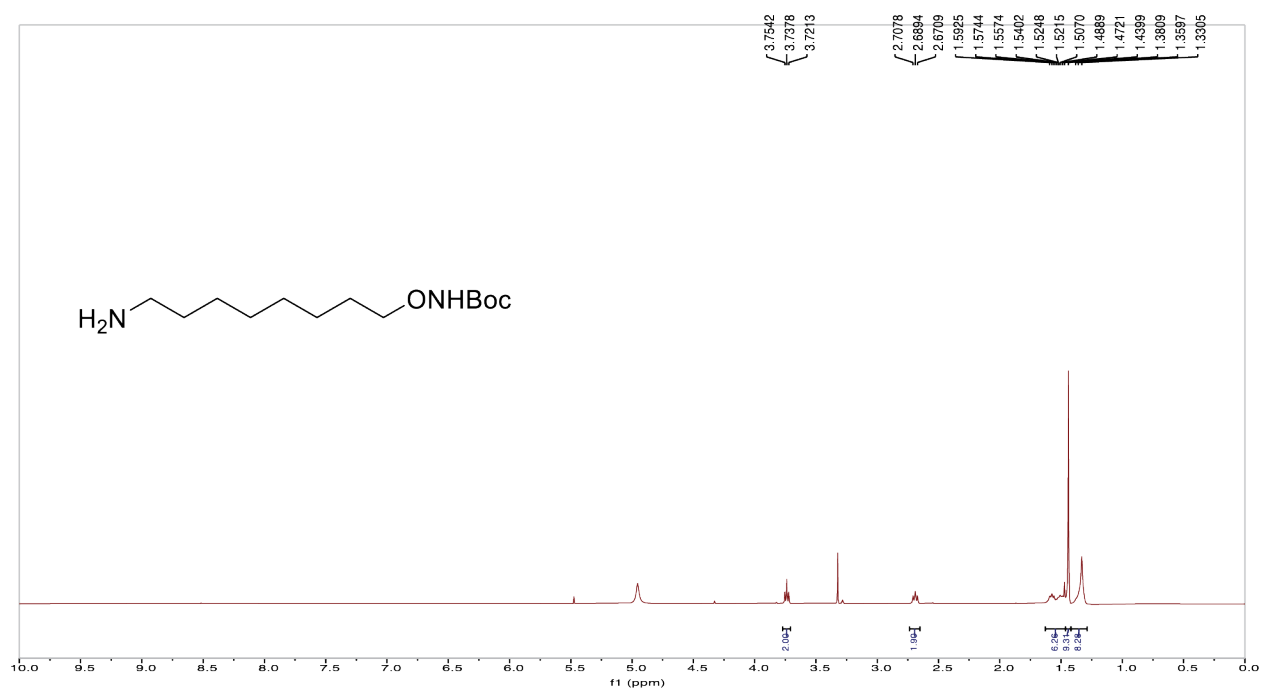


Figure S3.31. ¹H NMR Spectrum of compound S9 (400 MHz, MeOD)

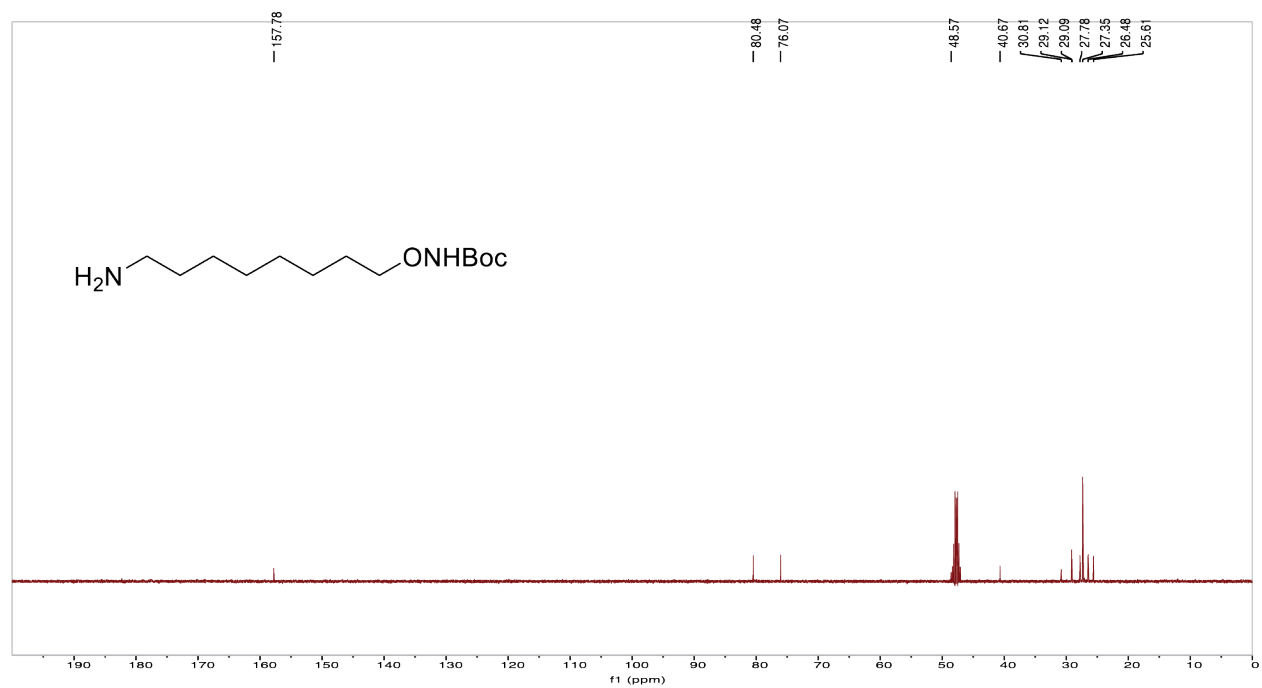


Figure 3.32. ¹³C NMR Spectrum of compound S9 (100 MHz, MeOD)

3.9.5 NMR spectra of compound S4

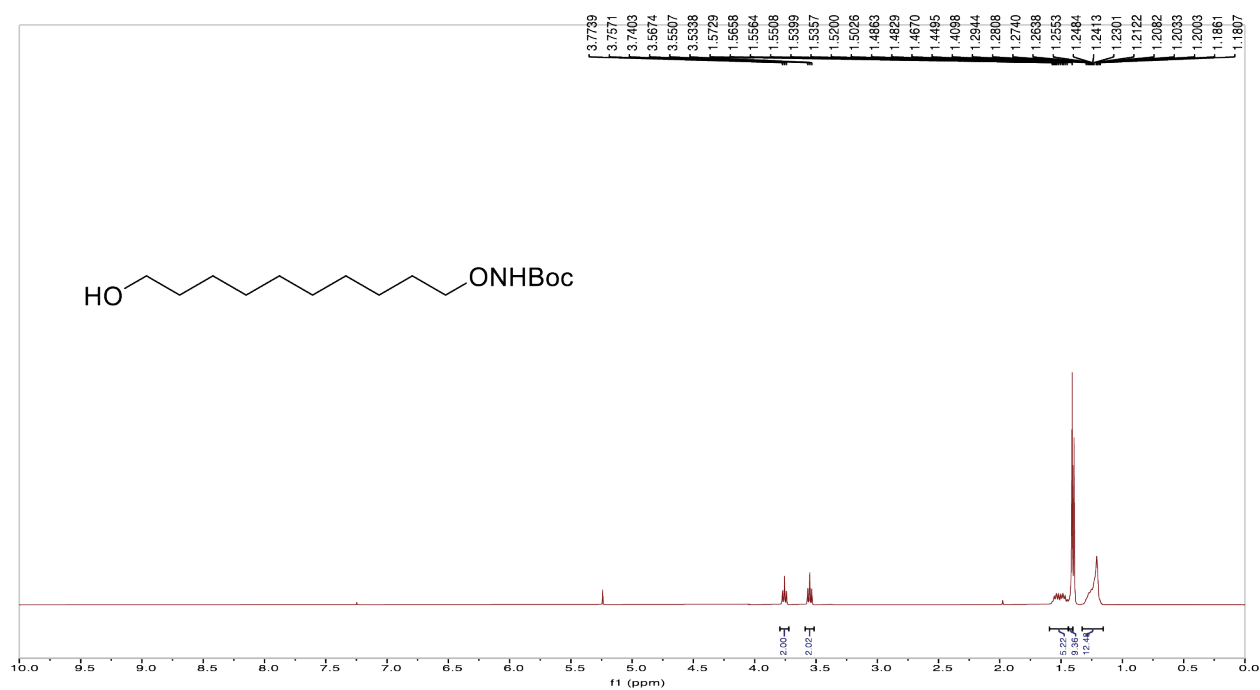


Figure S3.33. ¹H NMR spectrum of compound S4 (400 MHz, CDCl₃)

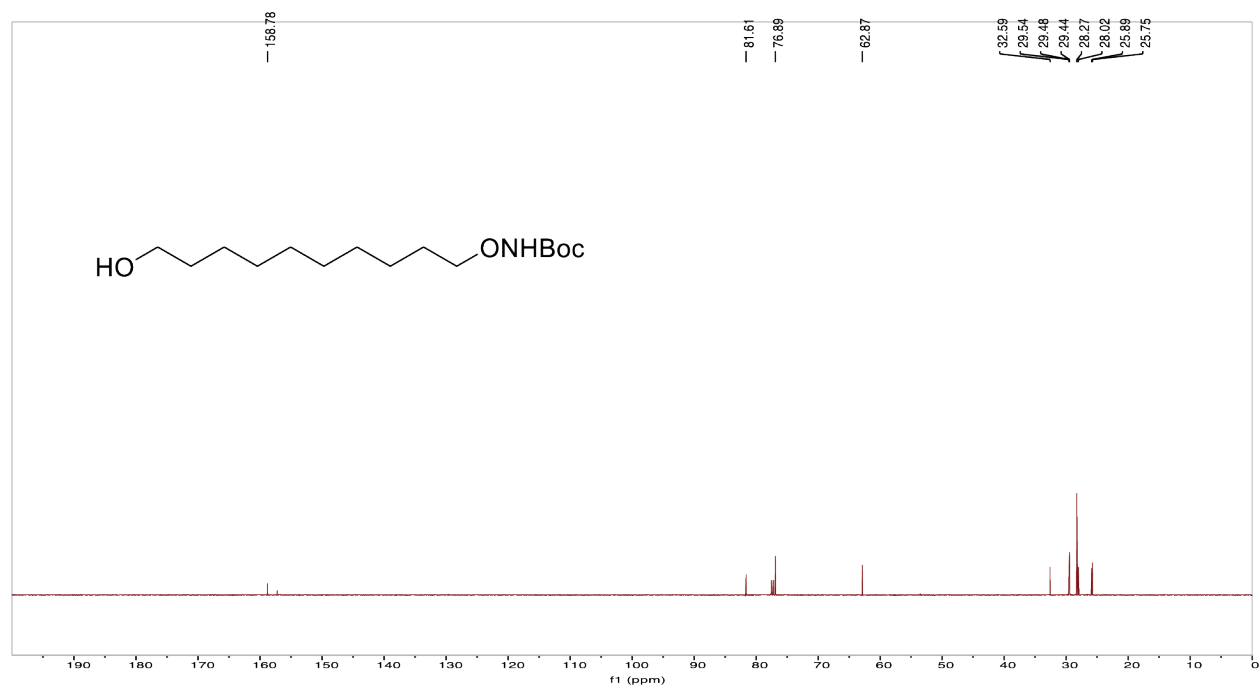


Figure S3.34. ¹³C NMR spectrum of compound S4 (100 MHz, CDCl₃)

3.9.6 NMR spectra of compound S6

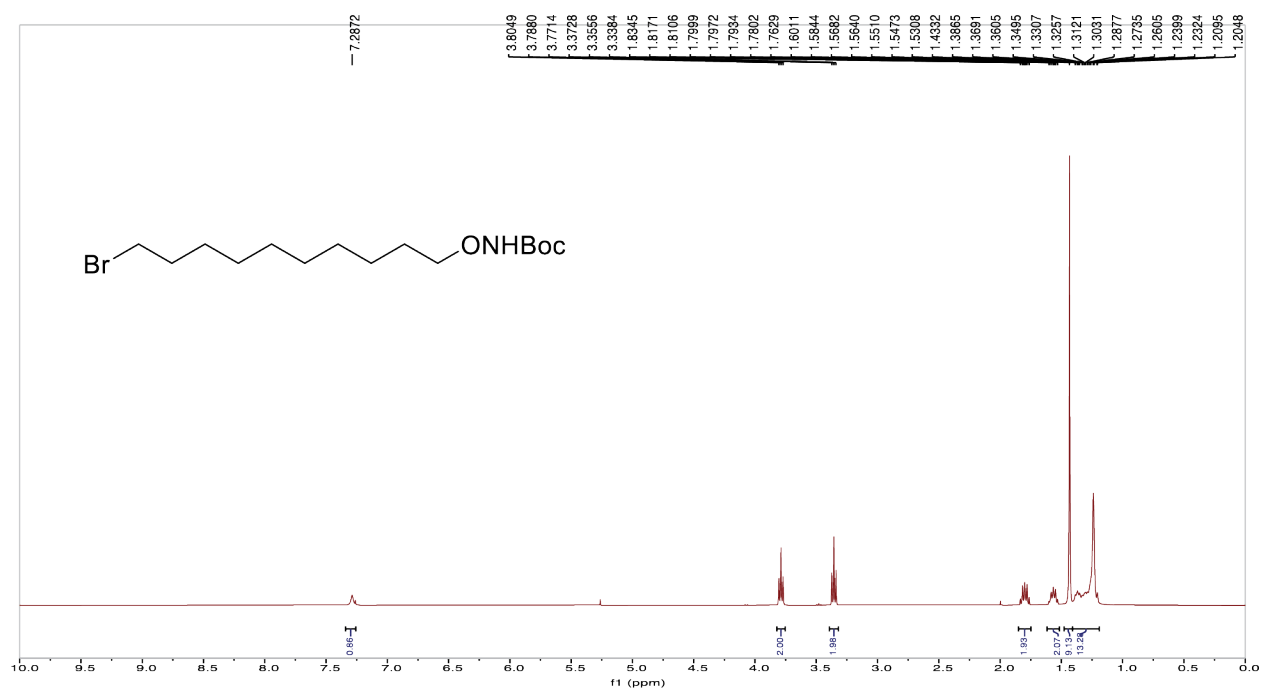


Figure S3.35. ¹H NMR spectrum of compound S6 (400 MHz, CDCl₃)

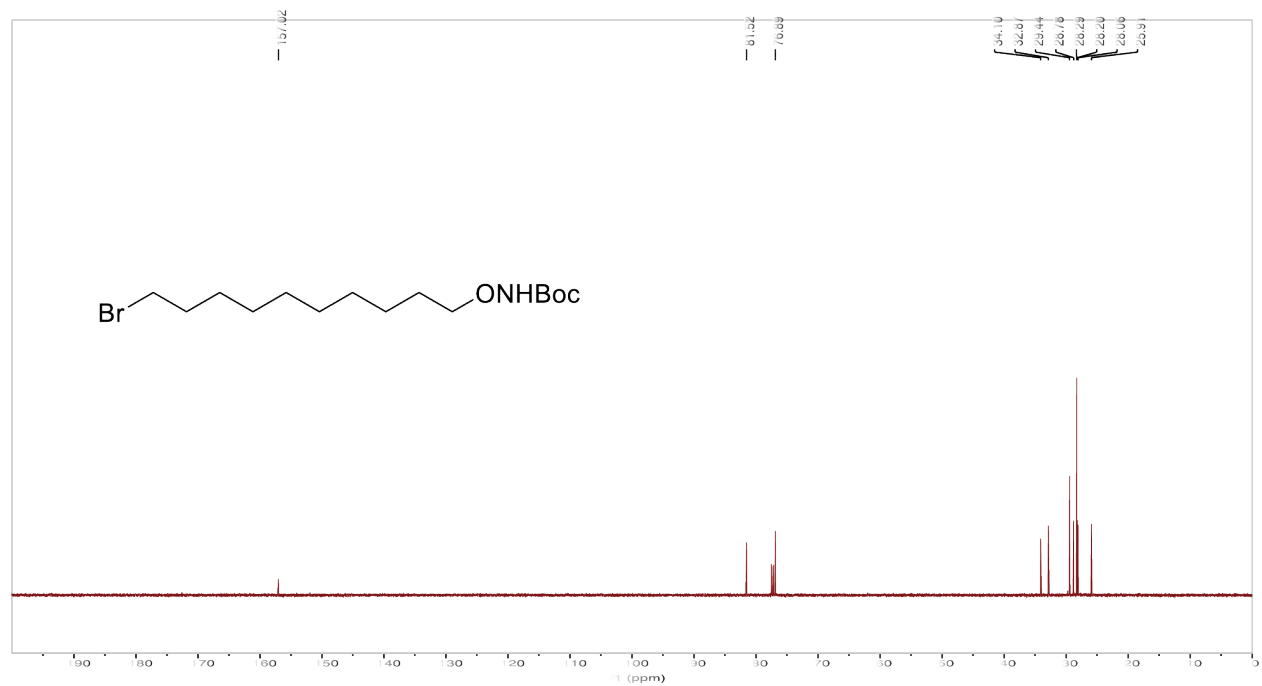


Figure S3.36. ¹³C NMR spectrum of compound S6 (100 MHz, CDCl₃)

3.9.7 NMR spectra of compound S8

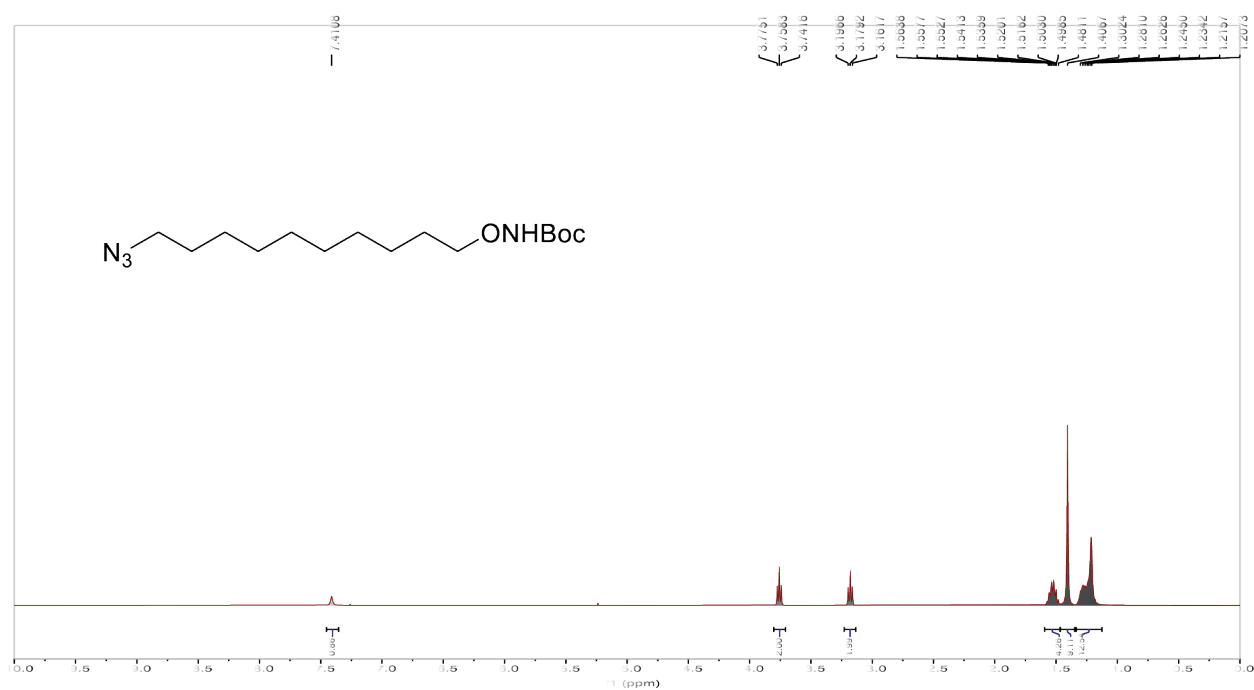


Figure S3.37. ¹H NMR spectrum of compound S8 (400 MHz, CDCl₃)

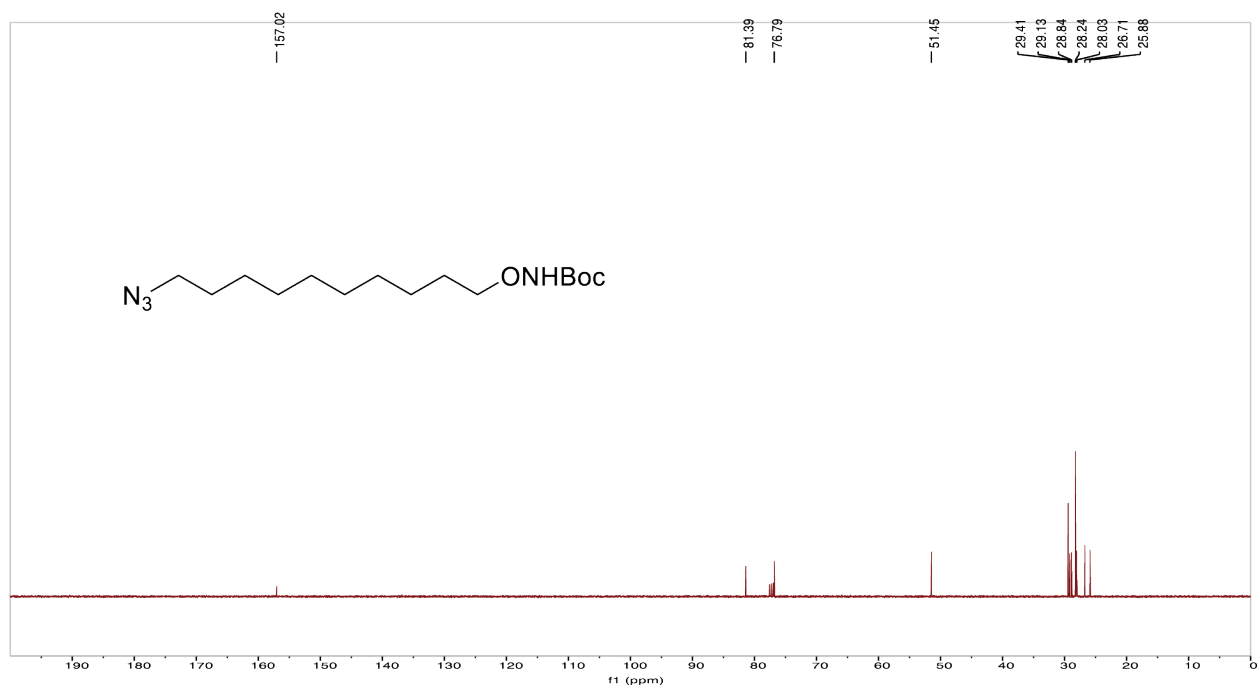


Figure S3.38. ¹³C NMR spectrum of compound S8 (100 MHz, CDCl₃)

3.9.8 NMR spectra of compound S10

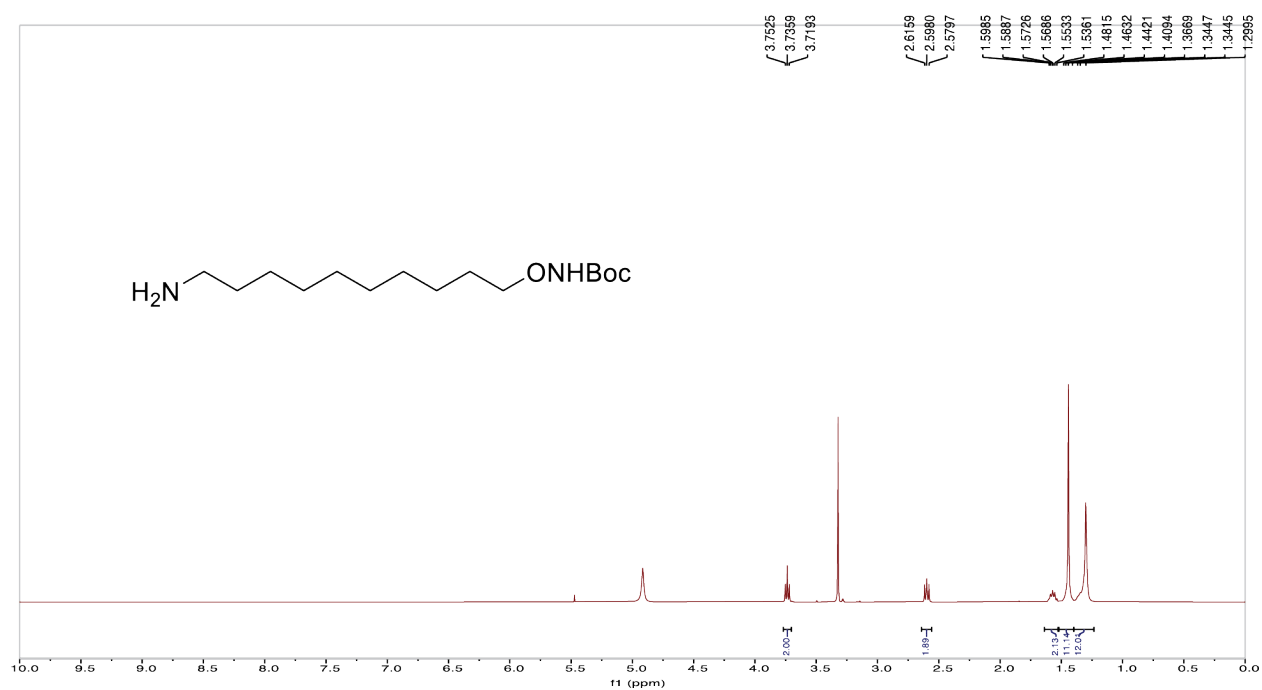


Figure S3.39. ¹H NMR spectrum of compound S10 (400 MHz, MeOD)

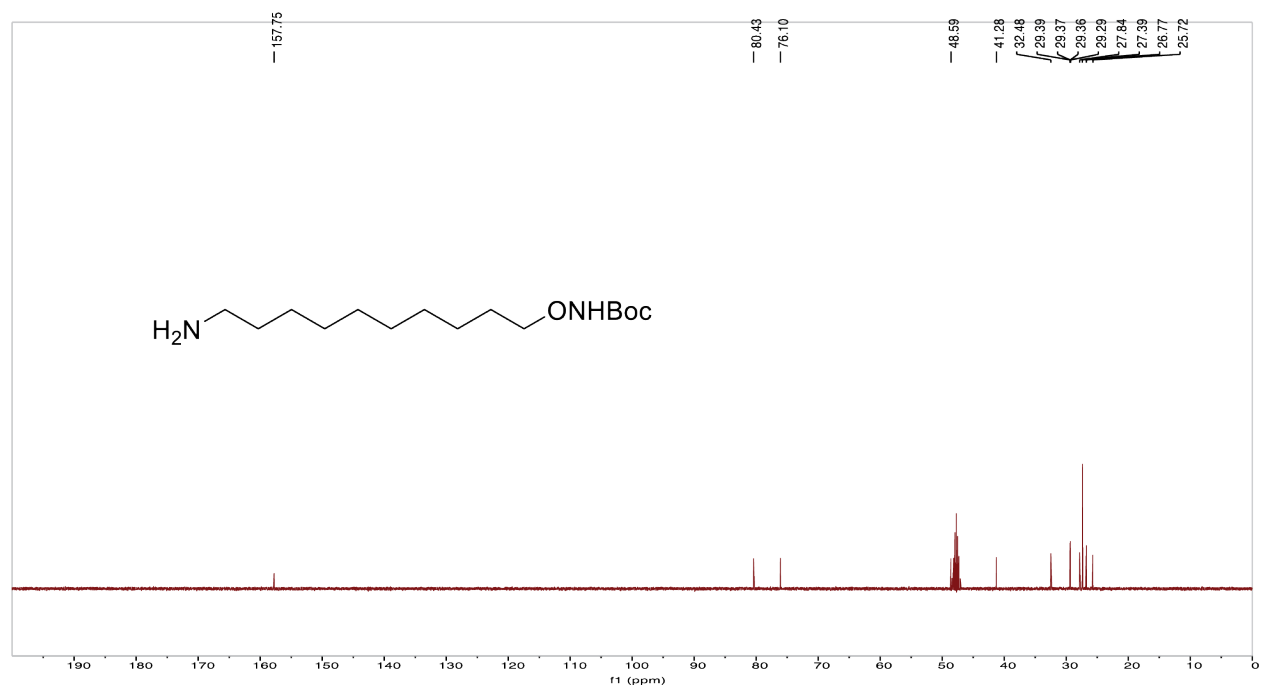


Figure S3.40. ¹³C NMR spectrum of compound S8 (100 MHz, CDCl₃)

3.9.9 NMR spectra of compound S11

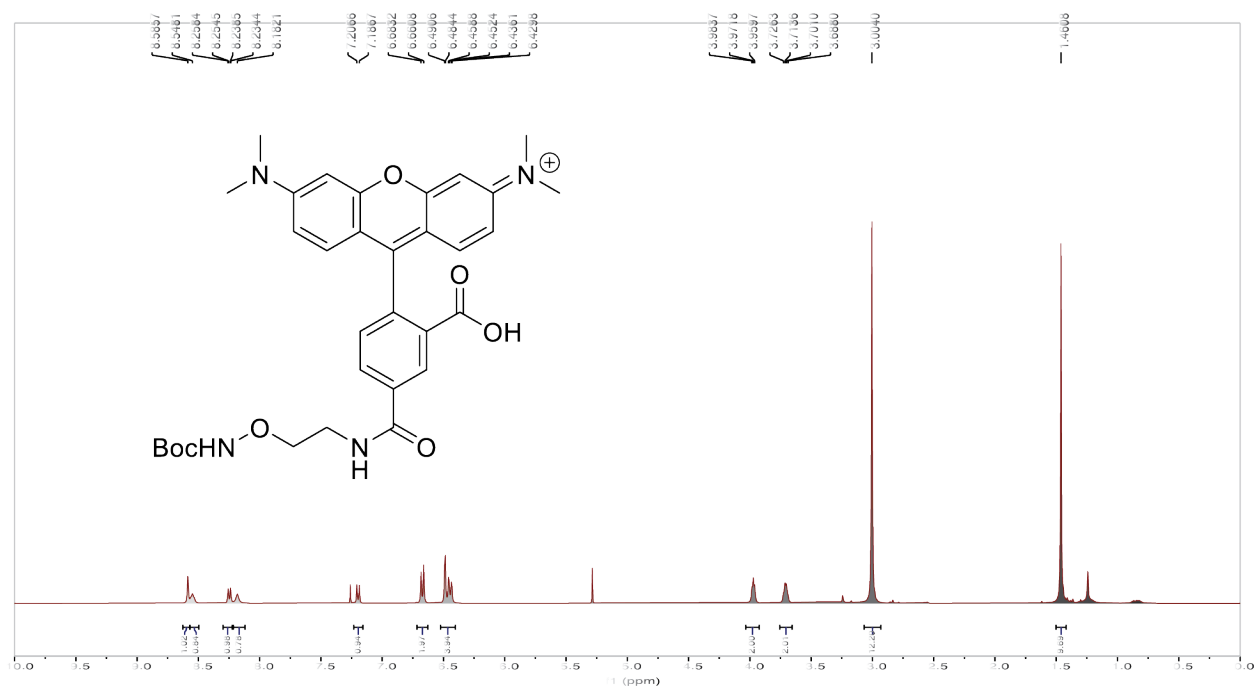


Figure S3.41. ¹H NMR spectrum of compound S11 (400 MHz, CDCl₃)

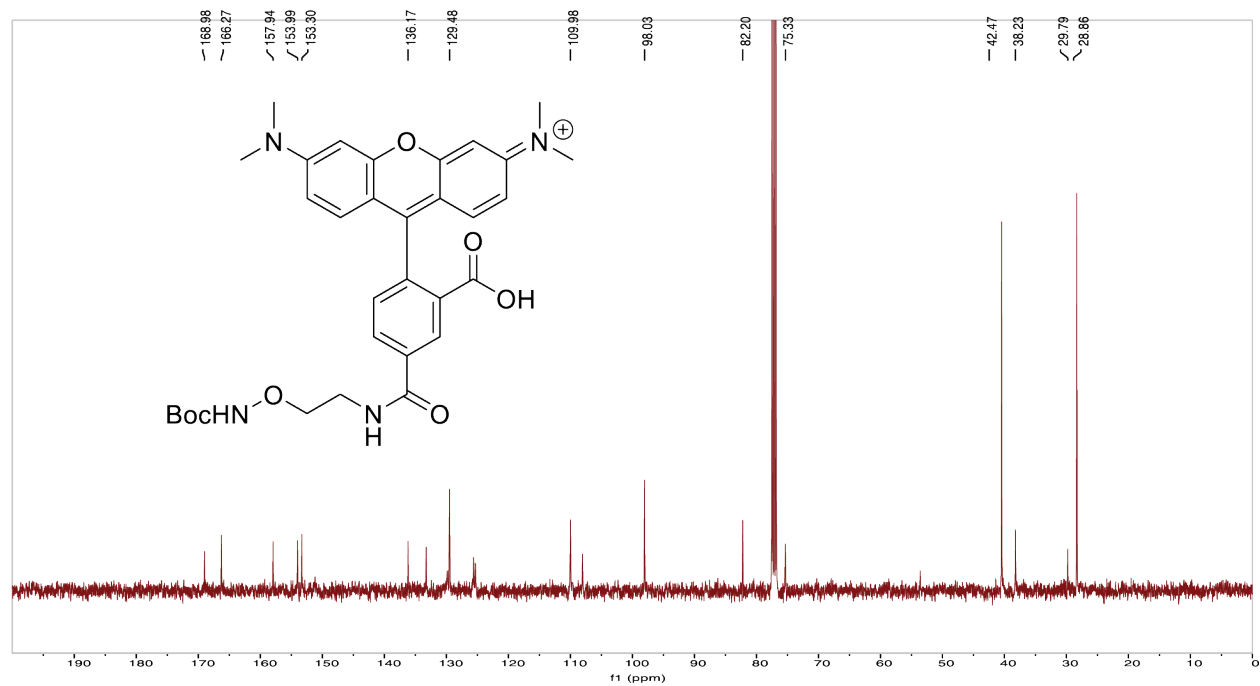


Figure S3.42. ¹³C NMR spectrum of compound S11 (100 MHz, CDCl₃)

3.9.10 NMR Spectra of compound S12

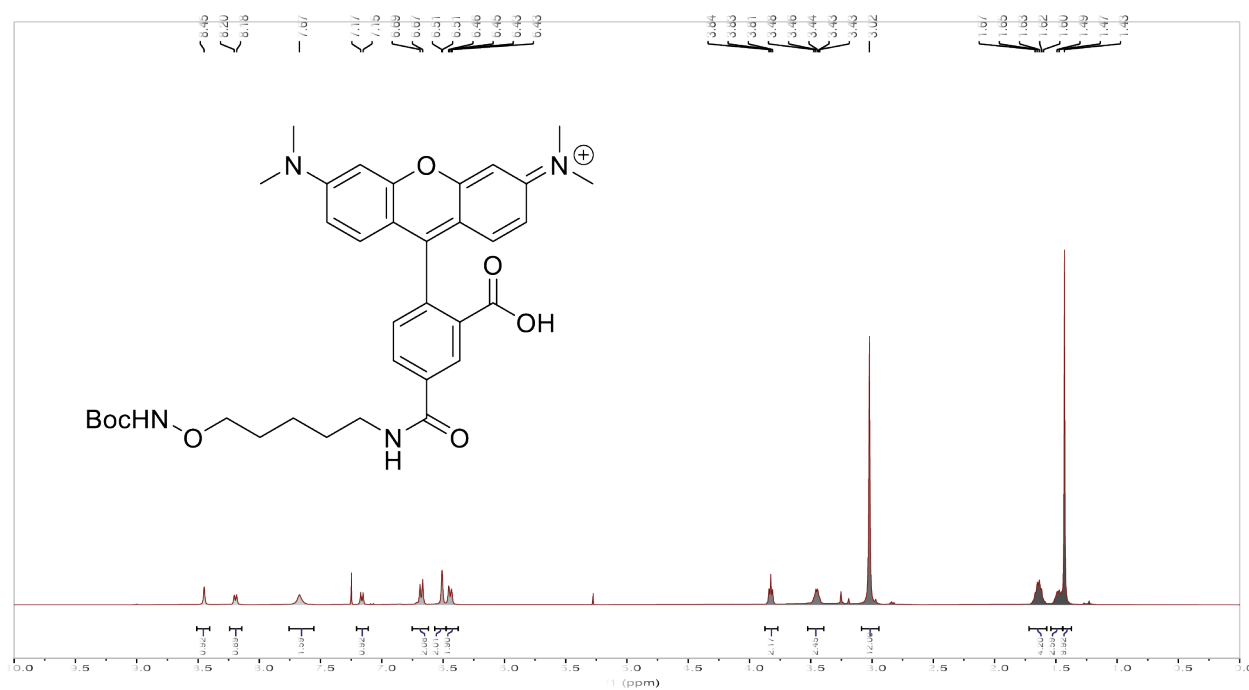


Figure S3.43. ^1H NMR spectrum of compound S12 (400 MHz, CDCl_3)

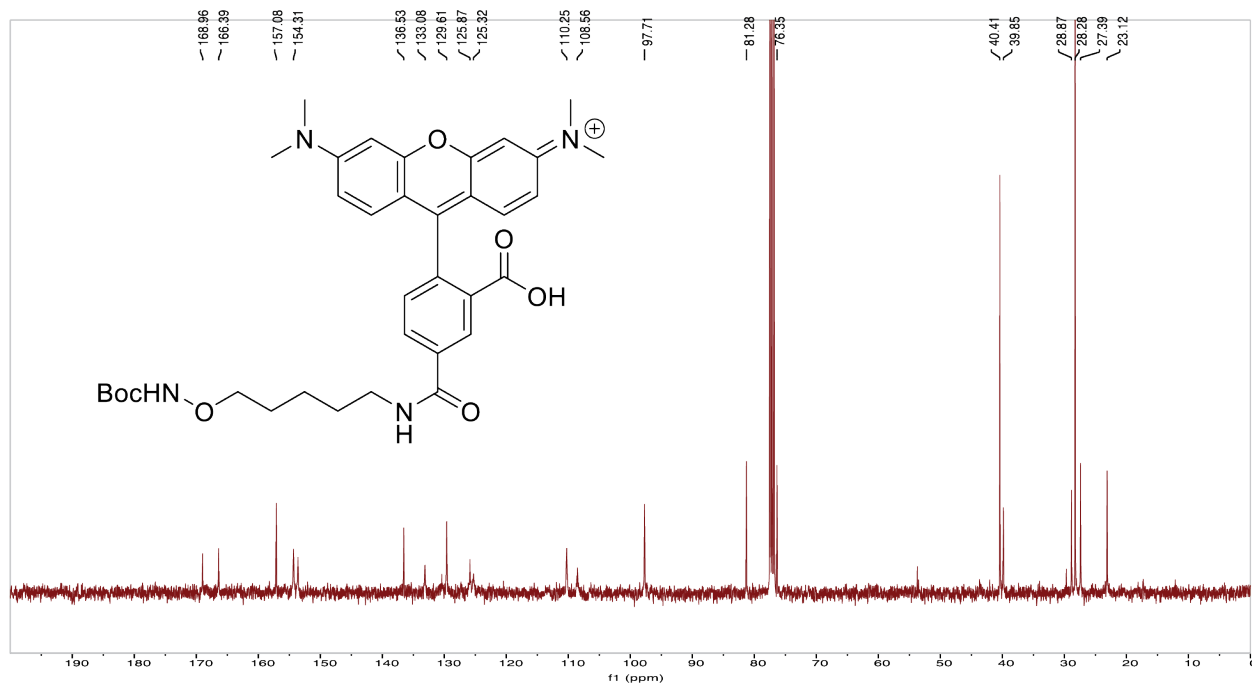


Figure S3.44. ^{13}C NMR spectrum of compound S12 (100 MHz, CDCl_3)

3.9.11 NMR spectra of compound S13

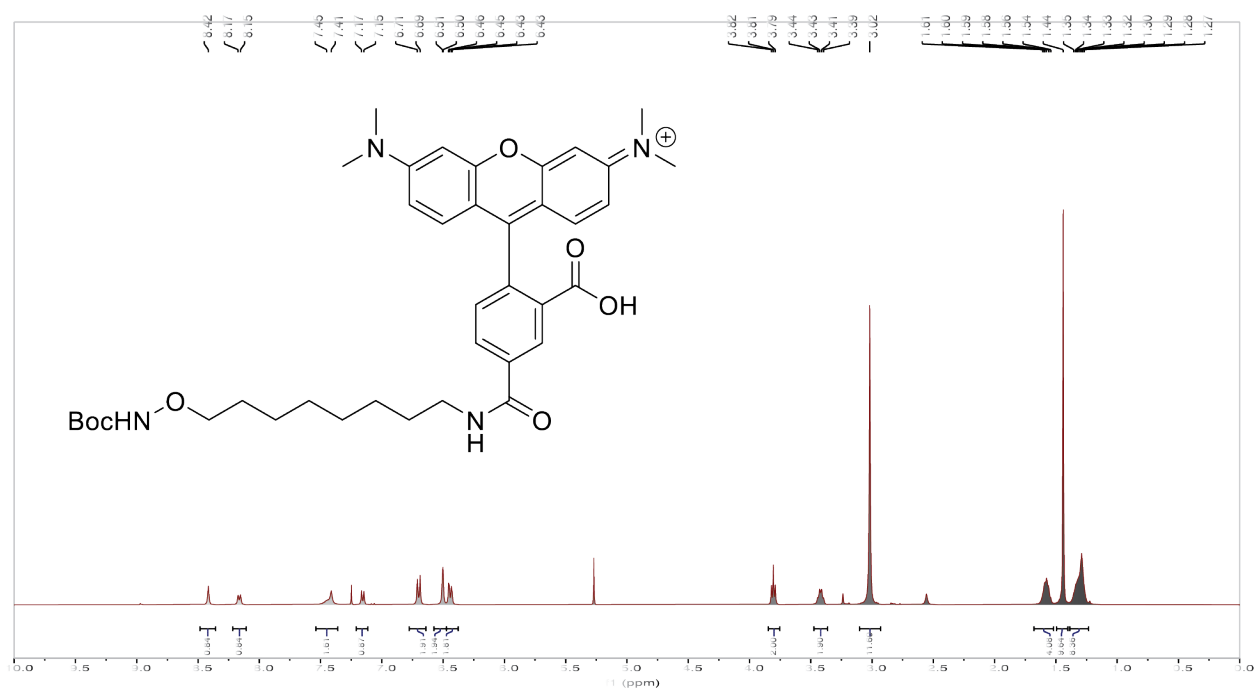


Figure S3.45. ¹H NMR spectrum of compound S13 (400 MHz, CDCl₃)

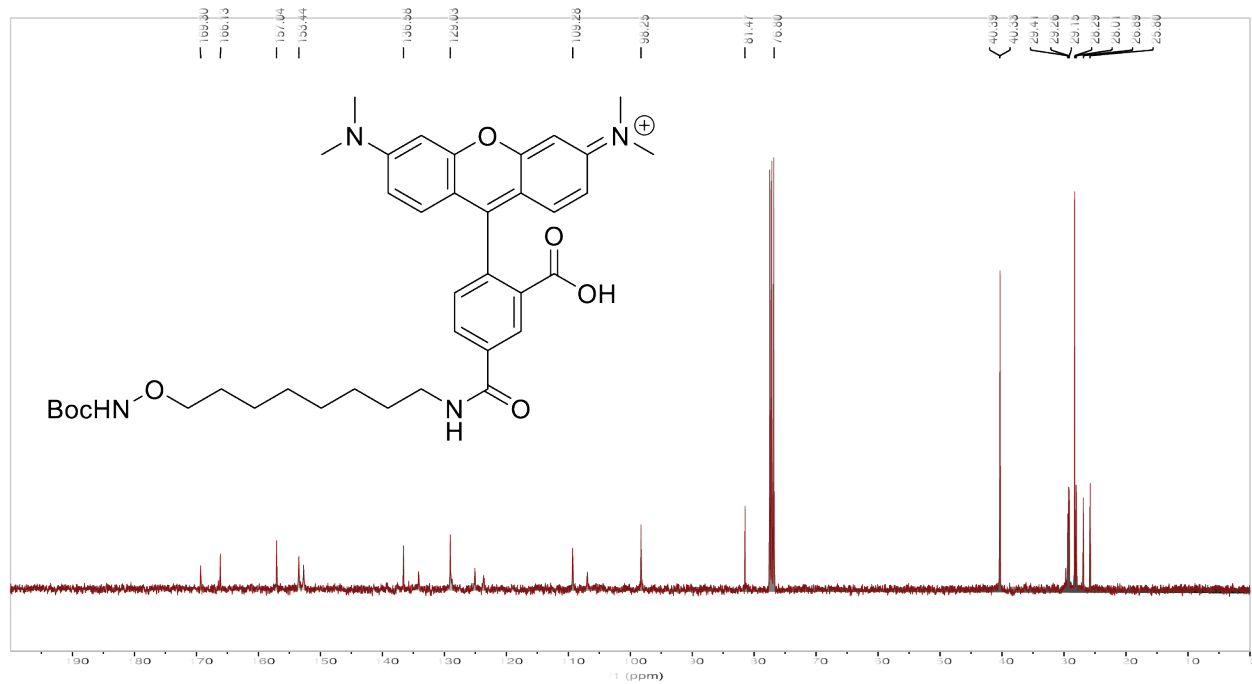


Figure S3.46. ¹³C NMR spectrum of compound S13 (100 MHz, CDCl₃)

3.9.12 NMR spectra of compound S14

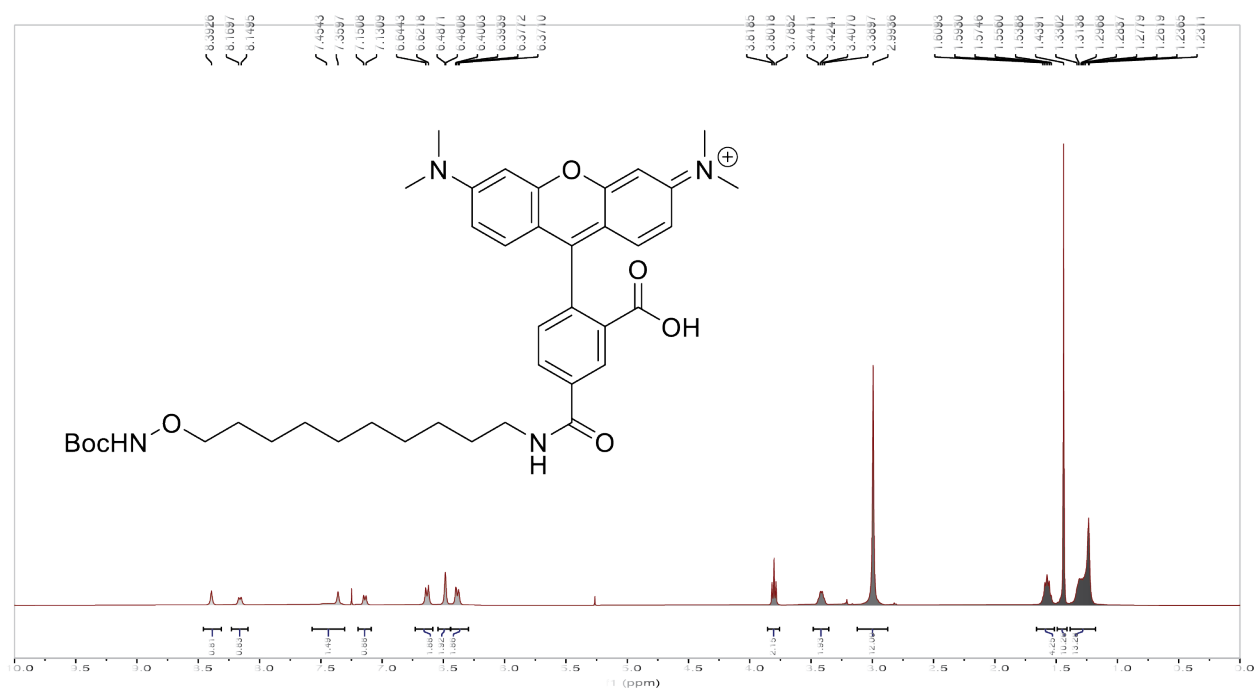


Figure S3.47. ¹H NMR spectrum of compound S14 (400 MHz, CDCl₃)

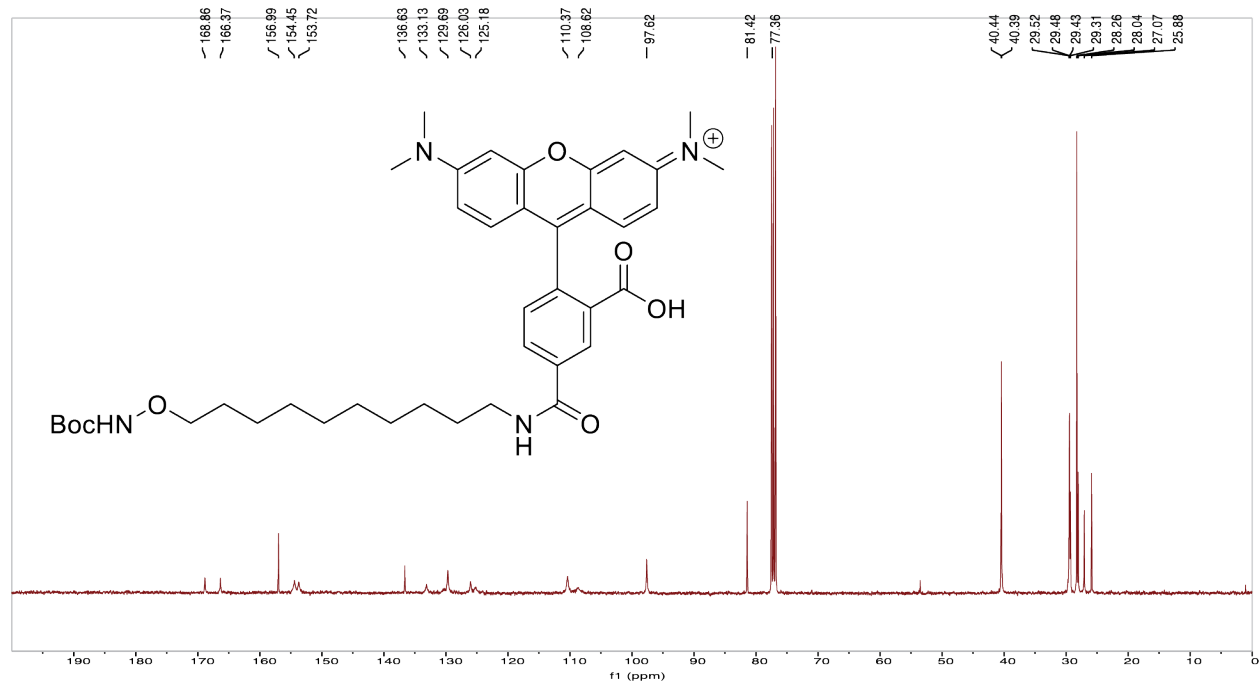


Figure S3.48. ¹³C NMR spectrum of compound S14 (100 MHz, CDCl₃)

3.9.13 NMR spectra of aminooxy-TAMRA 1

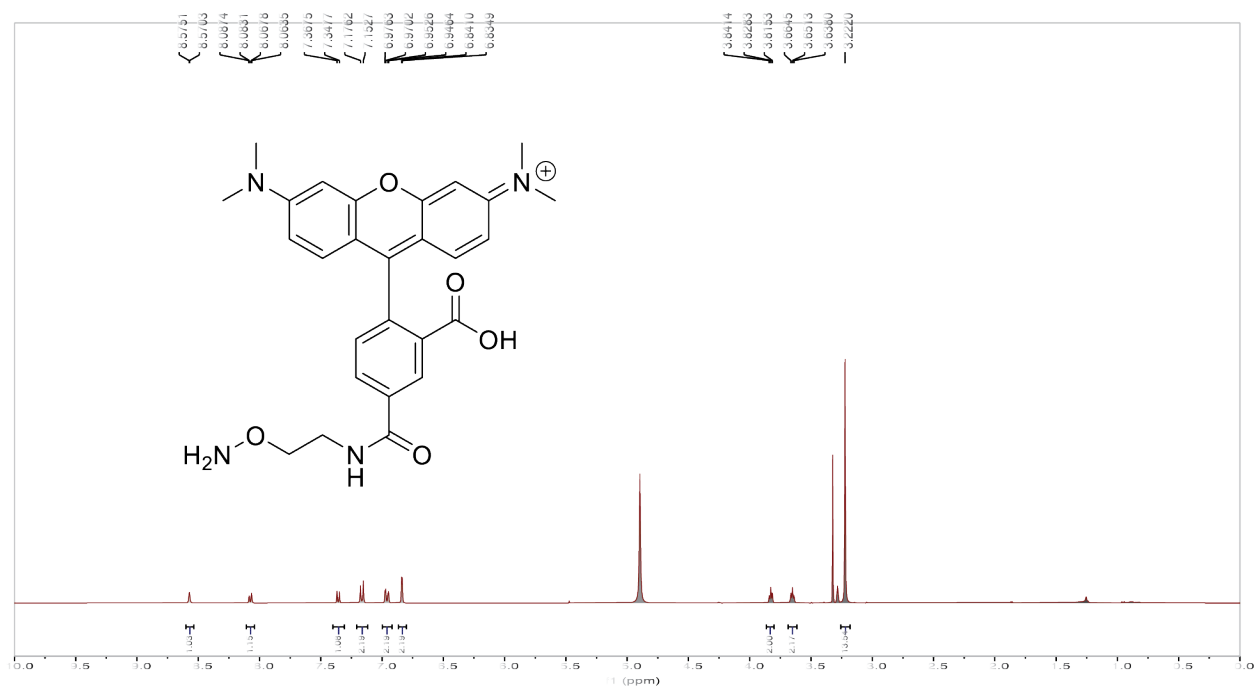


Figure S3.49. ^1H NMR spectrum of compound 1 (400 MHz, MeOD)

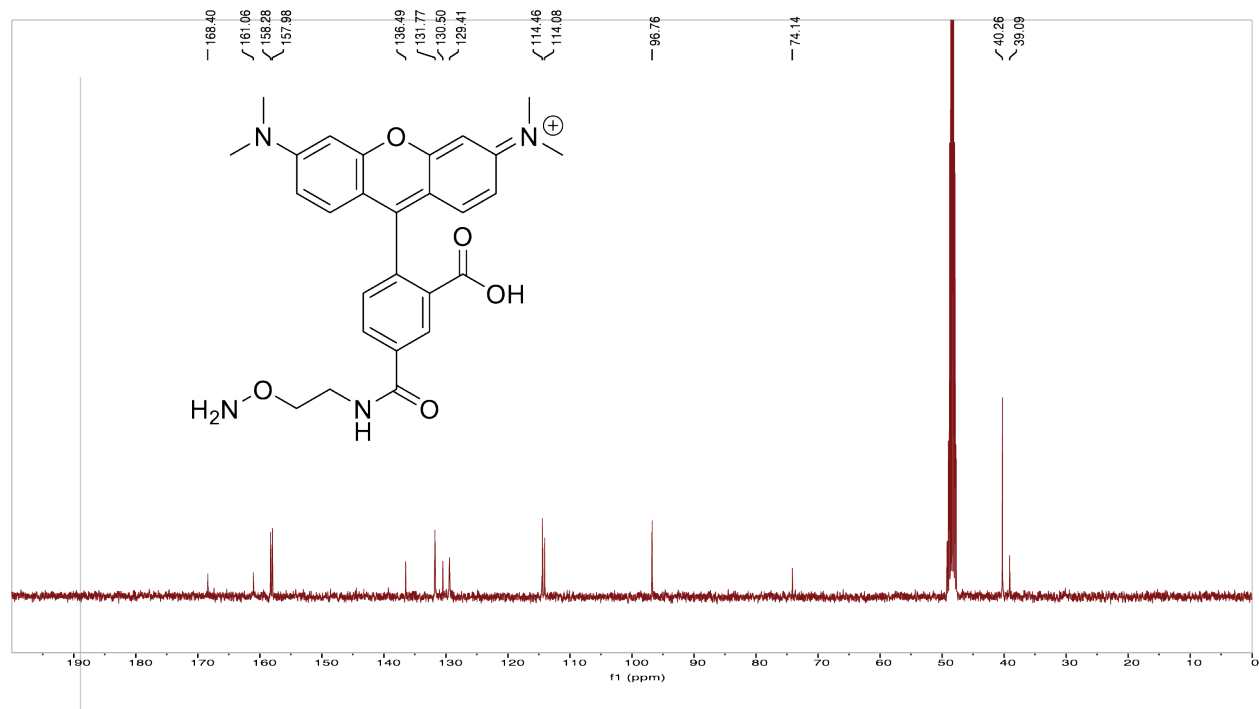


Figure S3.50. ^{13}C NMR spectrum of compound 1 (100 MHz, MeOD)

3.9.14 NMR spectra of aminoxy-TAMRA 2

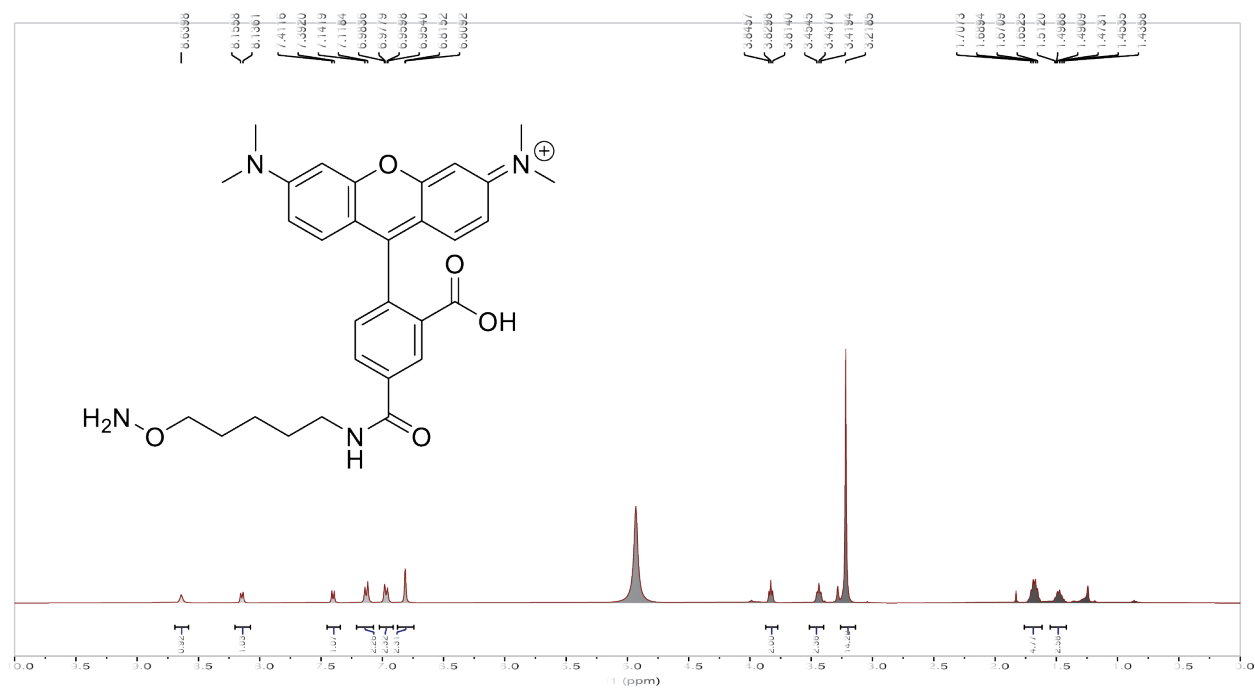


Figure S3.51. ¹H NMR spectrum of compound 2 (400 MHz, MeOD)

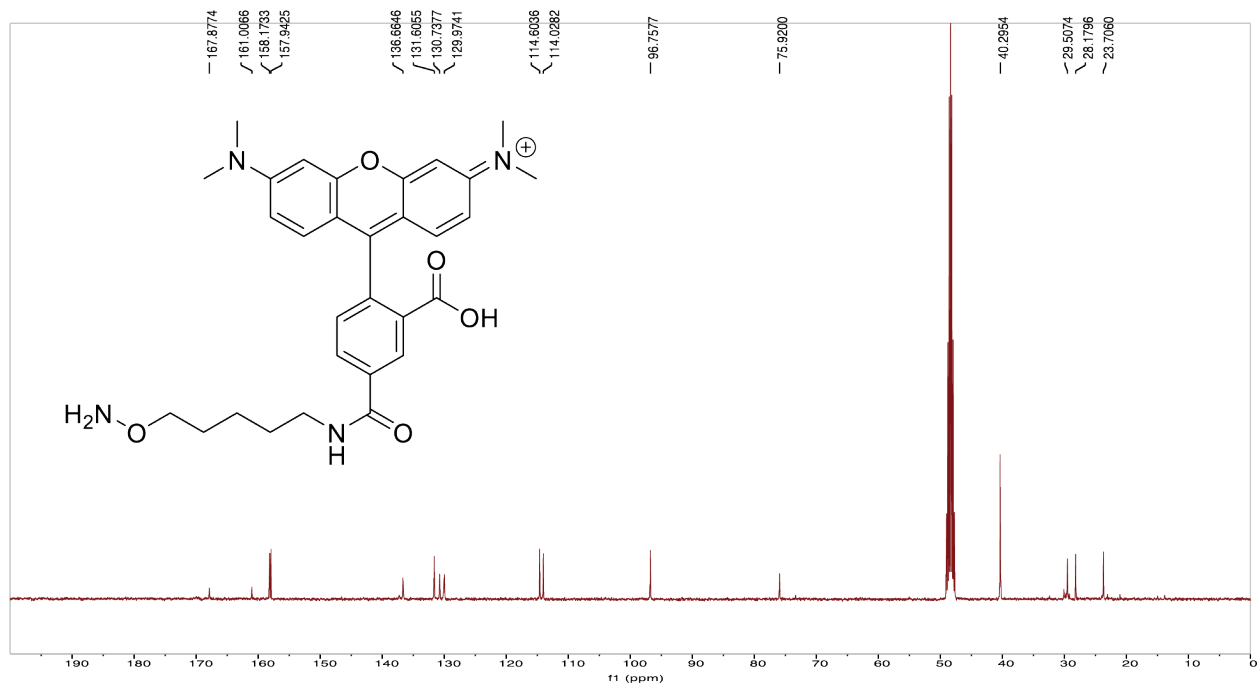


Figure S3.52. ¹³C NMR spectrum of compound 2 (100 MHz, MeOD)

3.9.15 NMR spectra of aminoxy-TAMRA 3

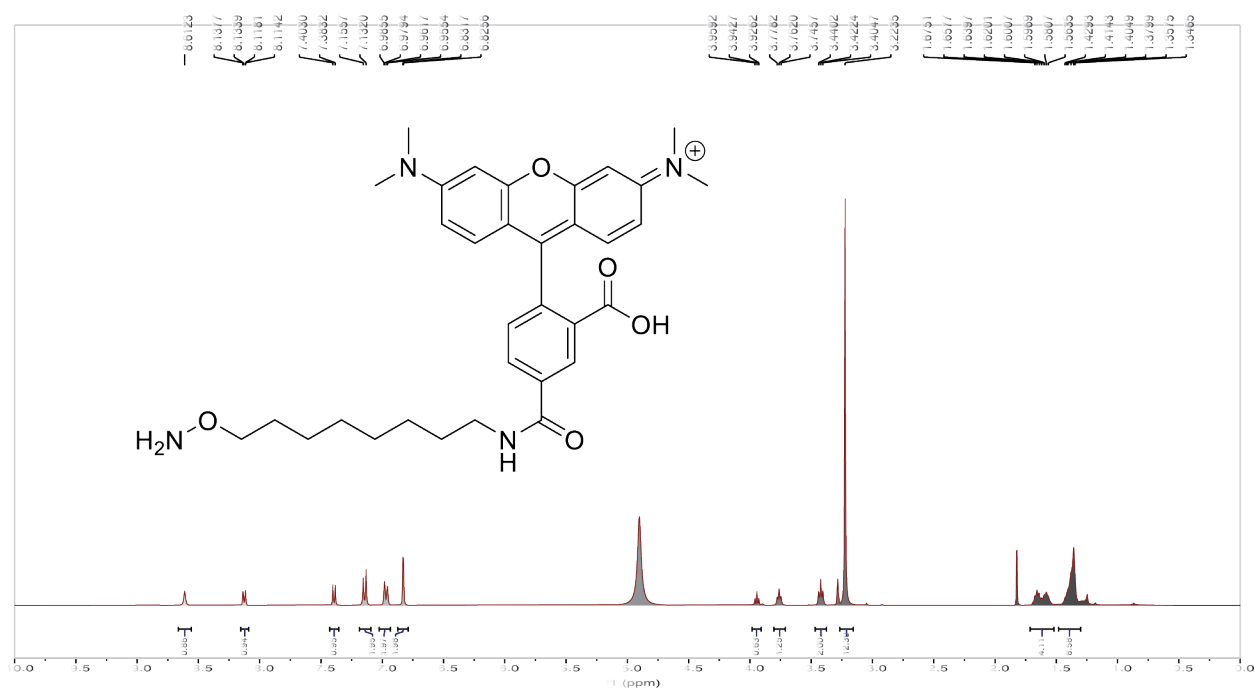


Figure S3.53. ¹H NMR spectrum of compound 3 (400 MHz, MeOD)

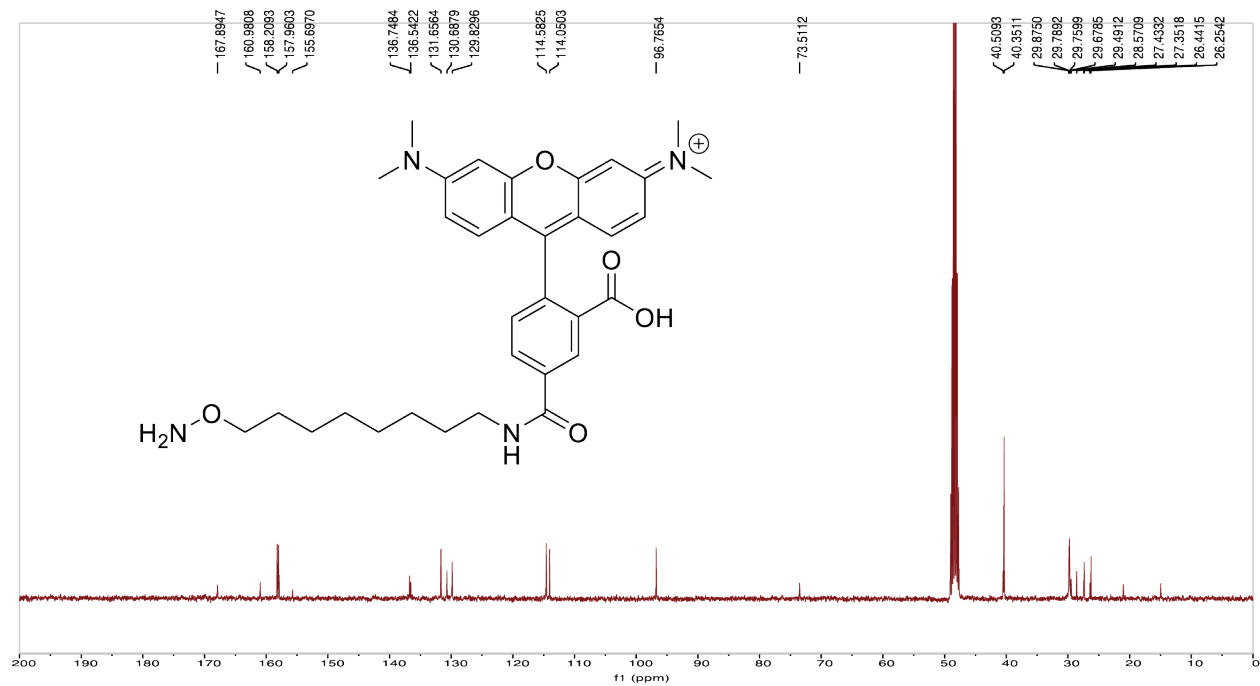


Figure S3.54. ¹³C NMR spectrum of compound 3 (100 MHz, MeOD)

3.9.16 NMR spectra of aminoxy-TAMRA 4

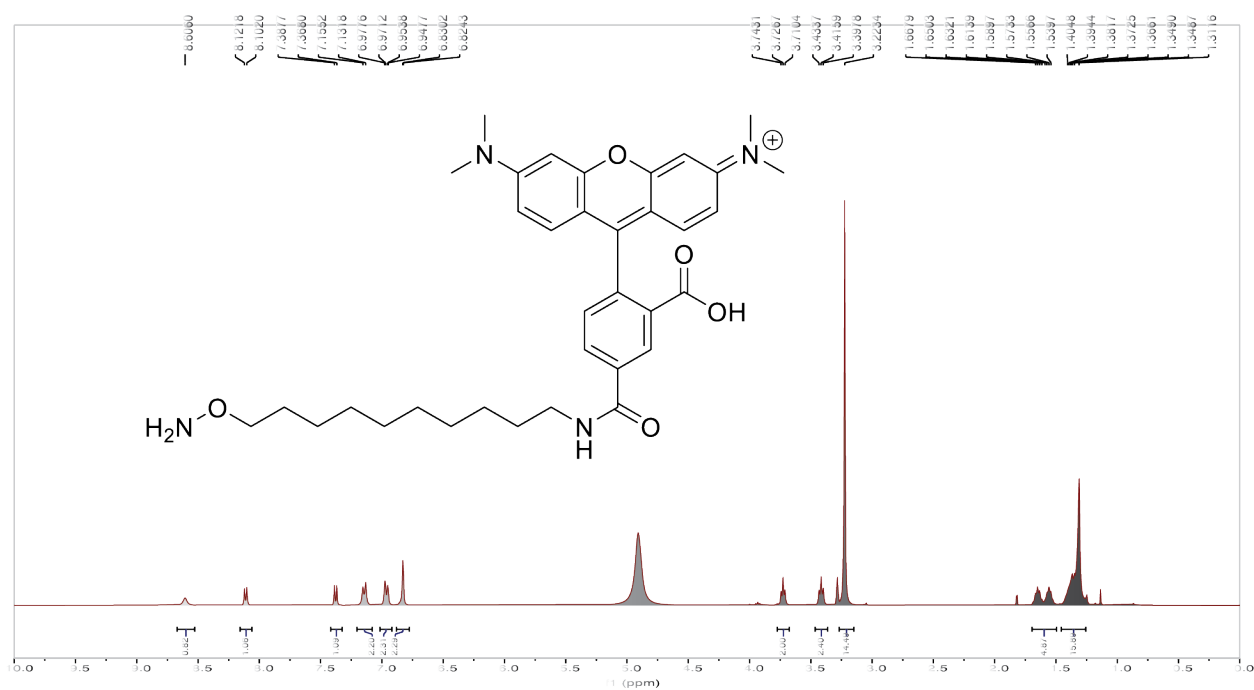


Figure S3.55. ¹H NMR spectrum of compound 4 (400 MHz, MeOD)

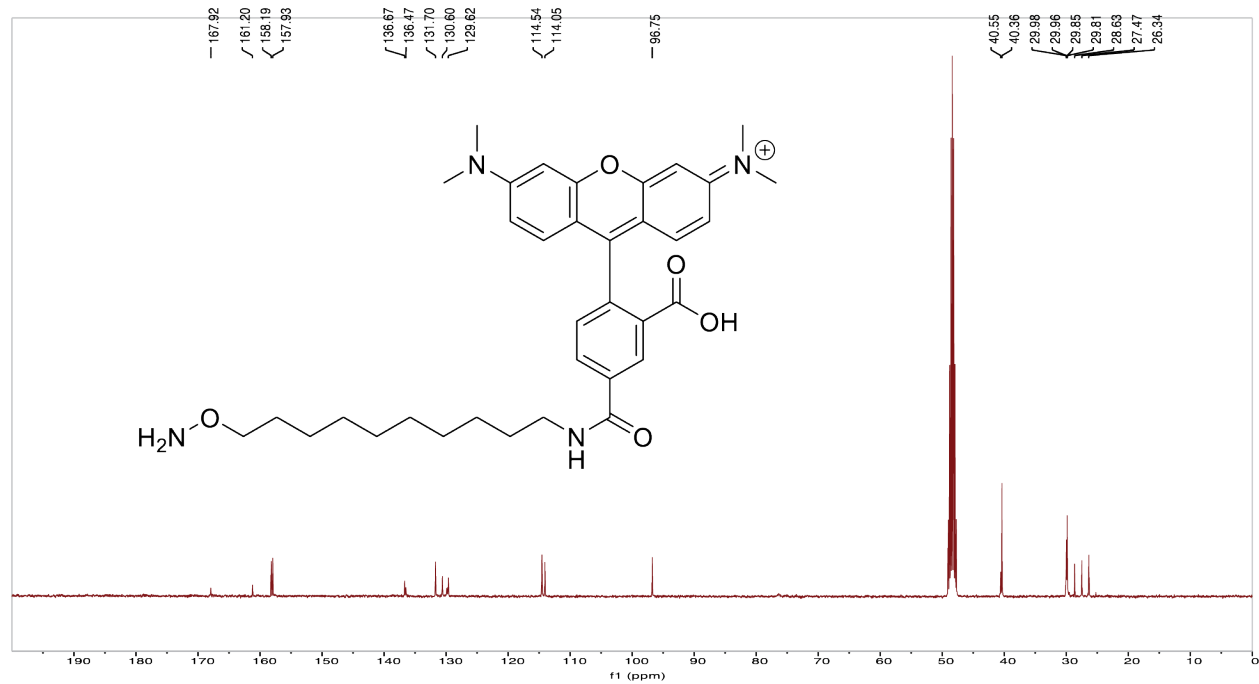


Figure S3.56. ¹³C NMR spectrum of compound 4 (100 MHz, MeOD)

3.9.17 NMR spectra of compound S19

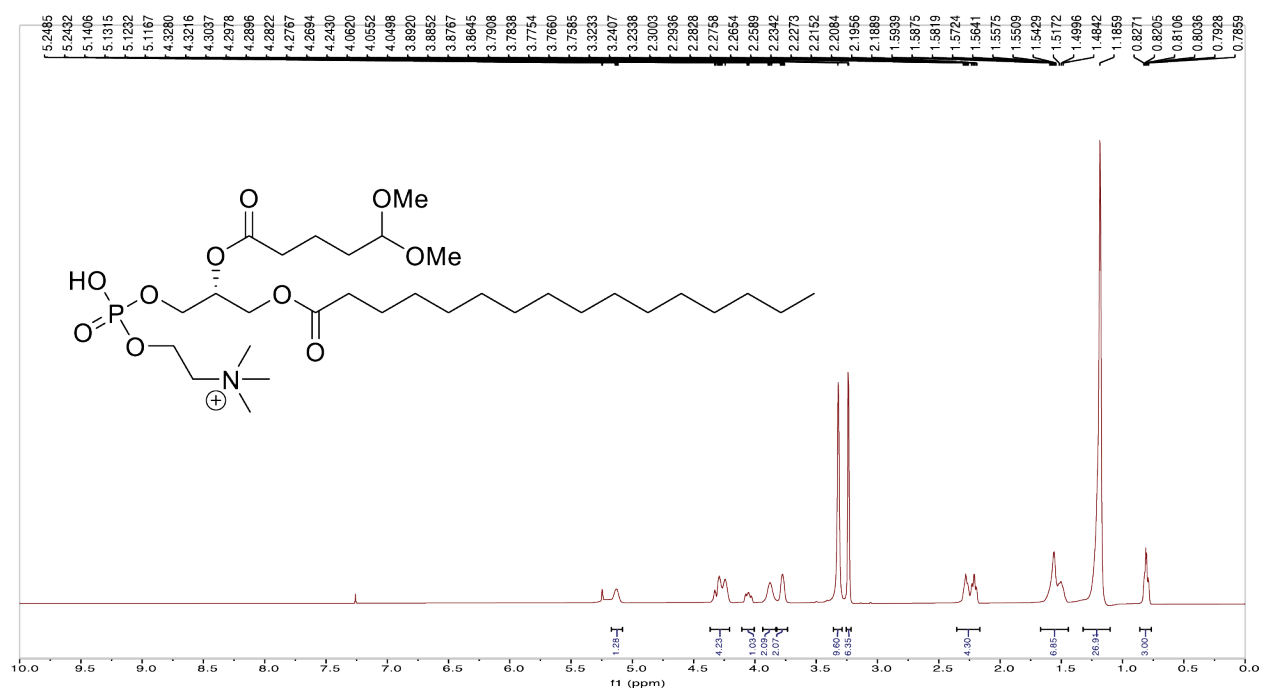


Figure S3.57. ^1H NMR spectrum of compound S19 (400 MHz, CDCl_3)

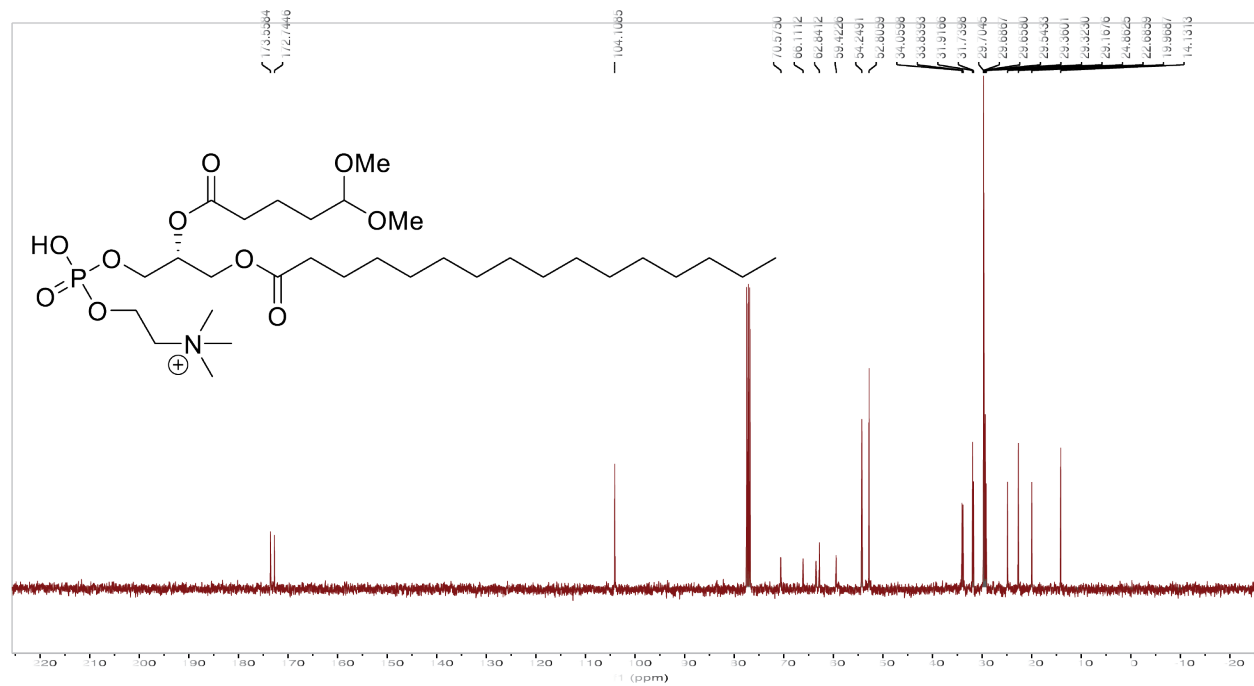


Figure S3.58. ^{13}C NMR spectrum of compound S19 (100 MHz, CDCl_3)

3.9.18 NMR spectra of POVPC

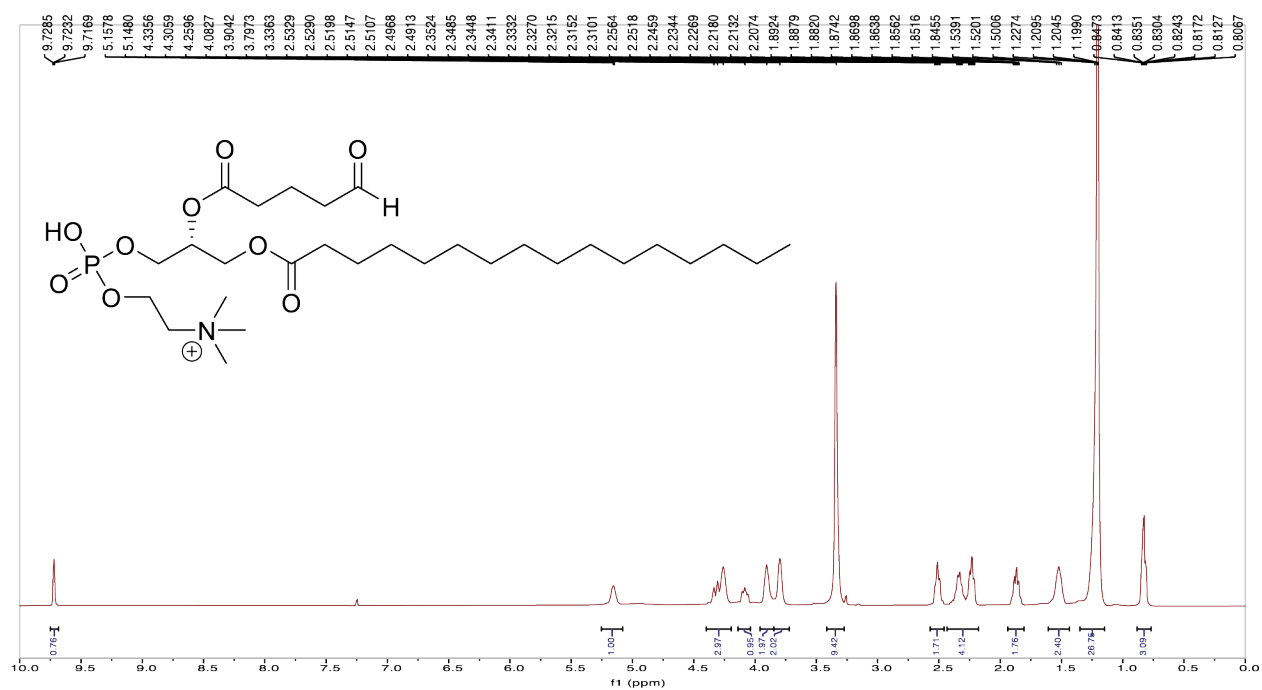


Figure S3.59. ¹H NMR spectrum of POVPC (400 MHz, CDCl₃)

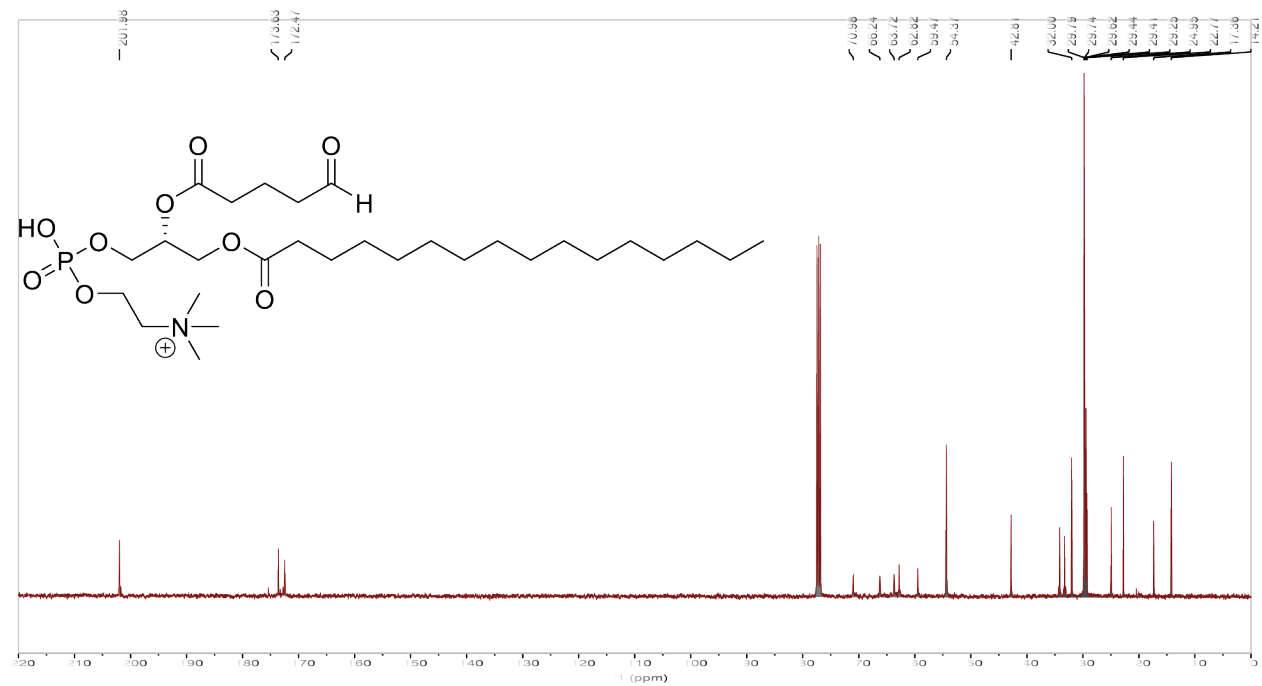


Figure S3.60. ¹³C NMR spectrum of POVPC (100 MHz, CDCl₃)

3.9.19 NMR spectra of PONPC

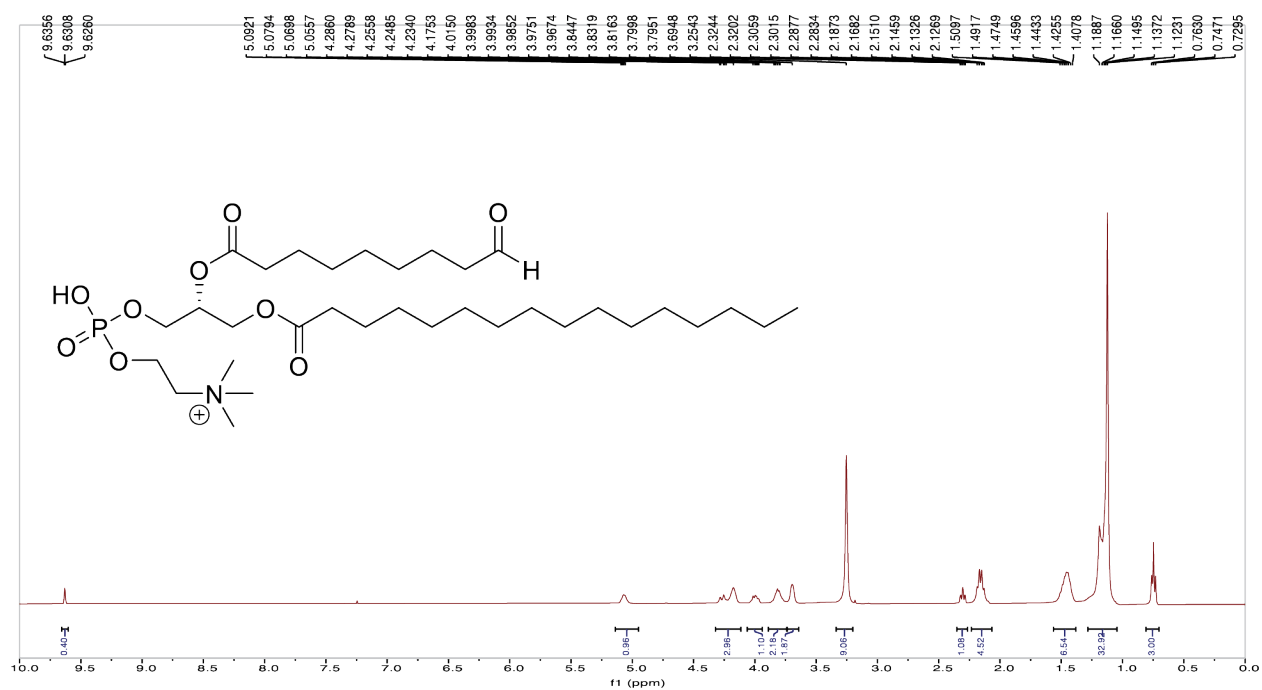


Figure S3.61. ^1H NMR spectrum of PONPC (400 MHz, CDCl_3)

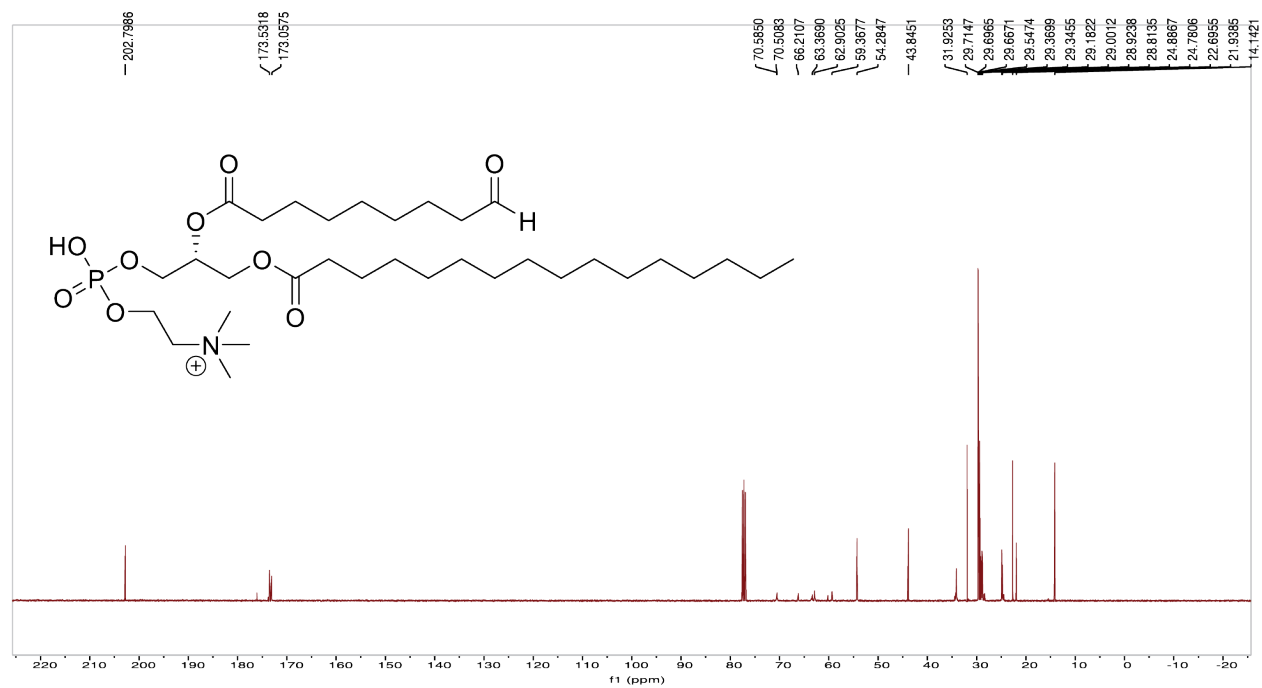


Figure S3.62. ^{13}C NMR spectrum of PONPC (100 MHz, CDCl_3)

3.9.20 NMR spectra of atheronal A

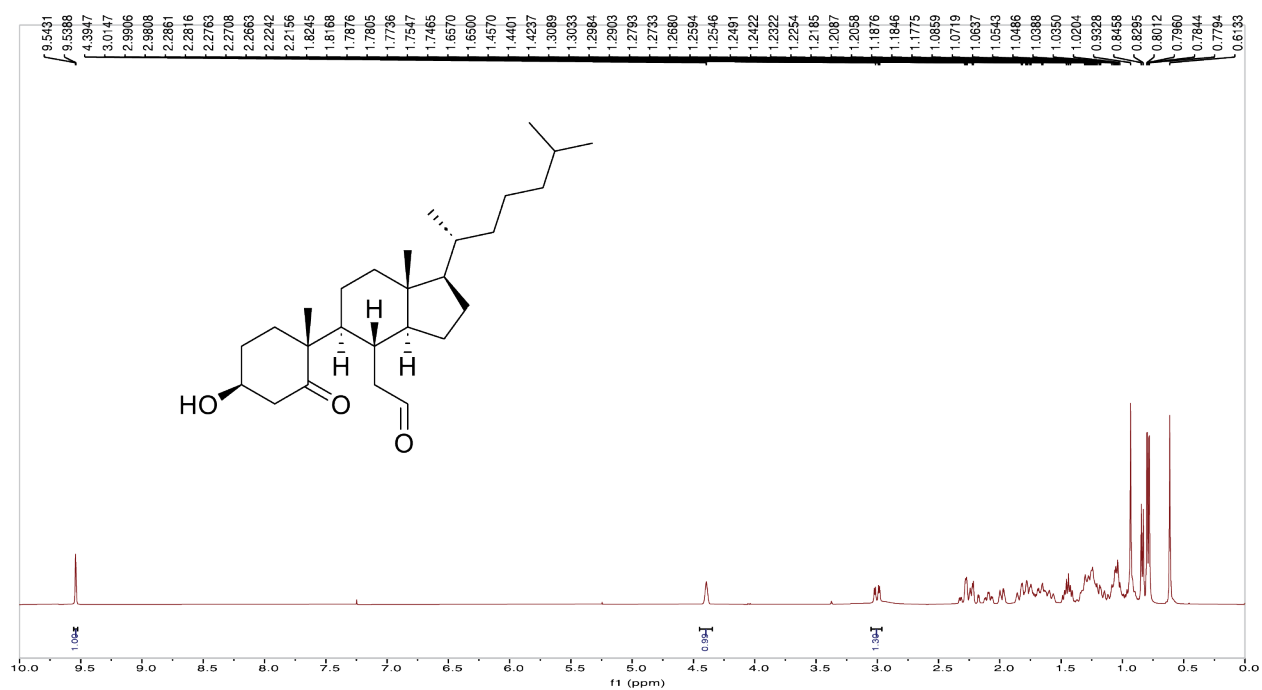


Figure S3.63. ¹H NMR spectrum of Atheronal A (400 MHz, CDCl₃)

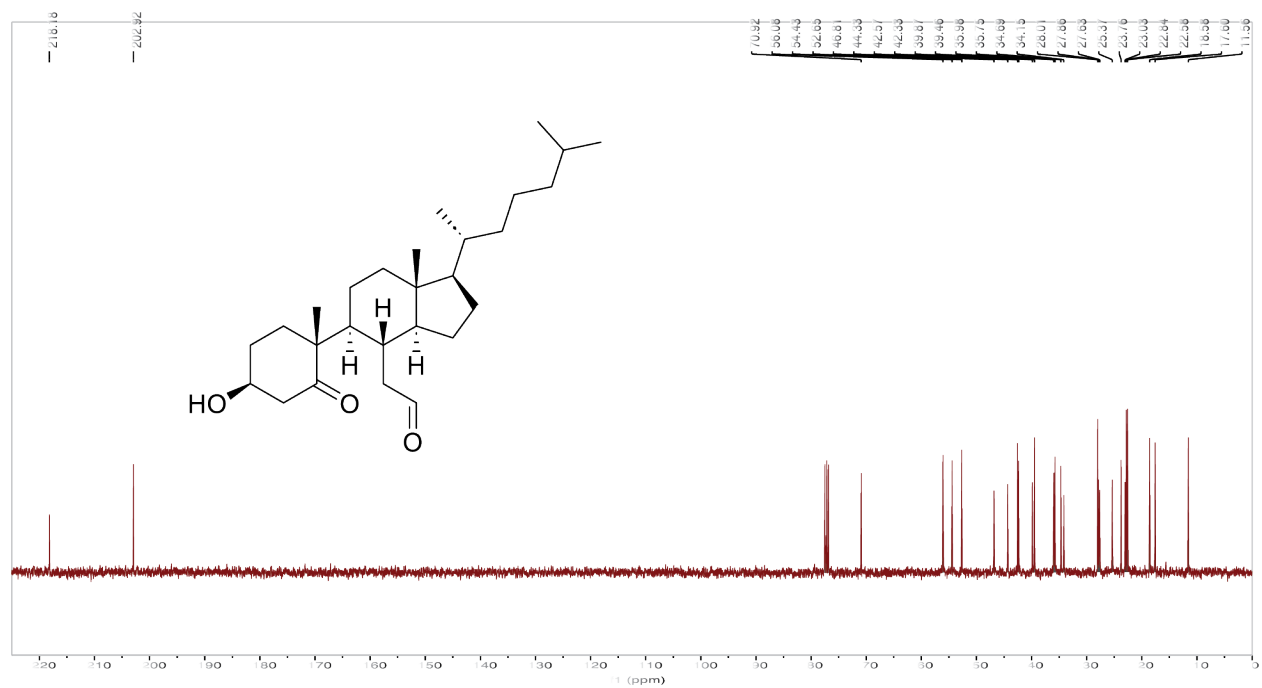


Figure S3.64. ¹³C NMR spectrum of Atheronal A (100 MHz, CDCl₃)

3.9.21 NMR spectra of 4-HNE

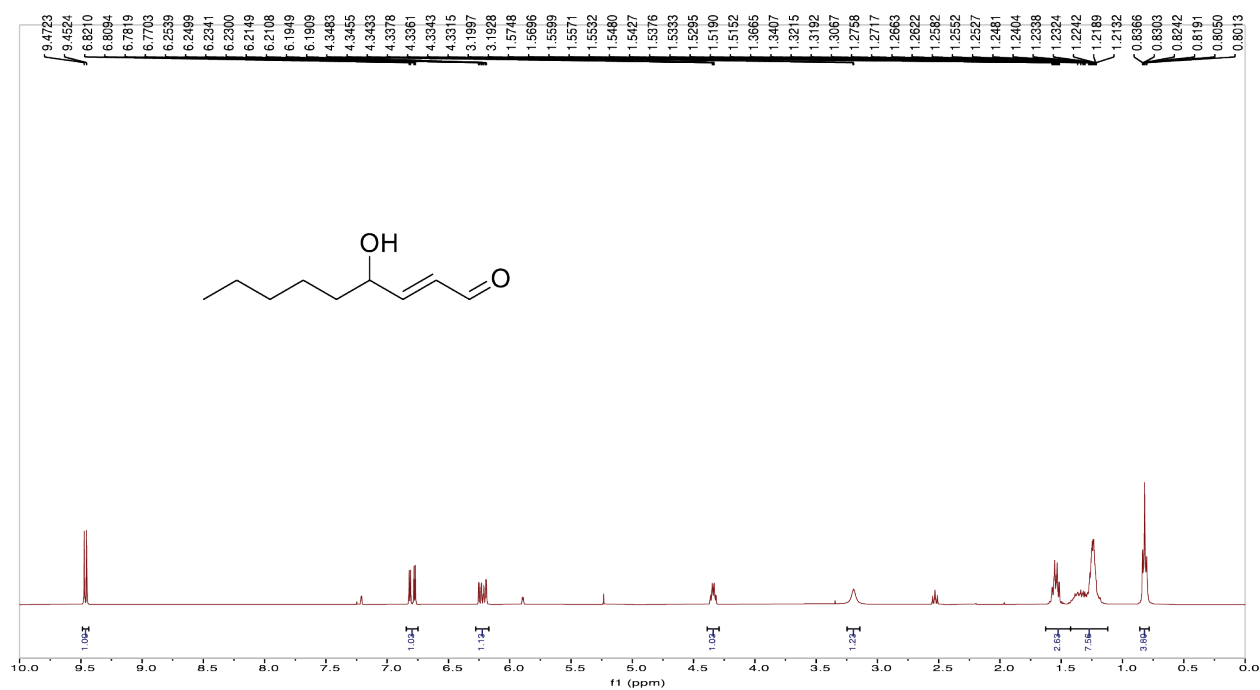


Figure S3.65. ¹H NMR spectrum of 4-HNE (400 MHz, CDCl₃)

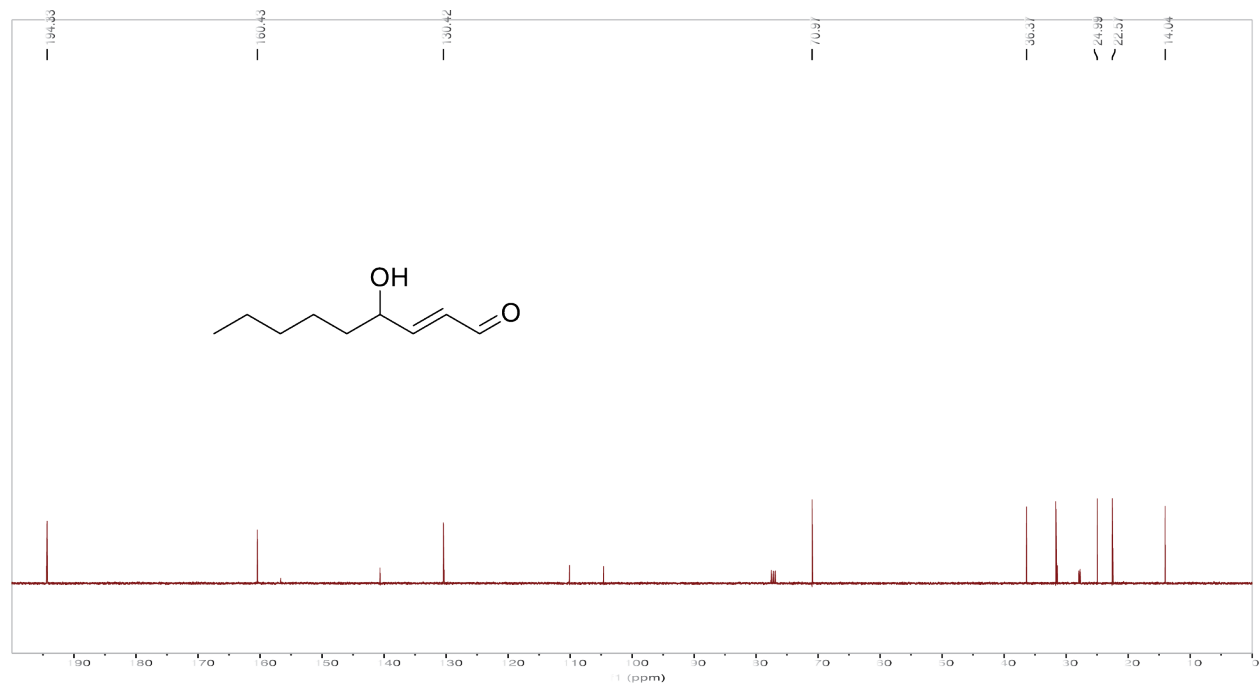


Figure S3.66. ¹³C NMR spectrum of compound 4-HNE (100 MHz, CDCl₃)

3.9.22 NMR spectra of compound 9

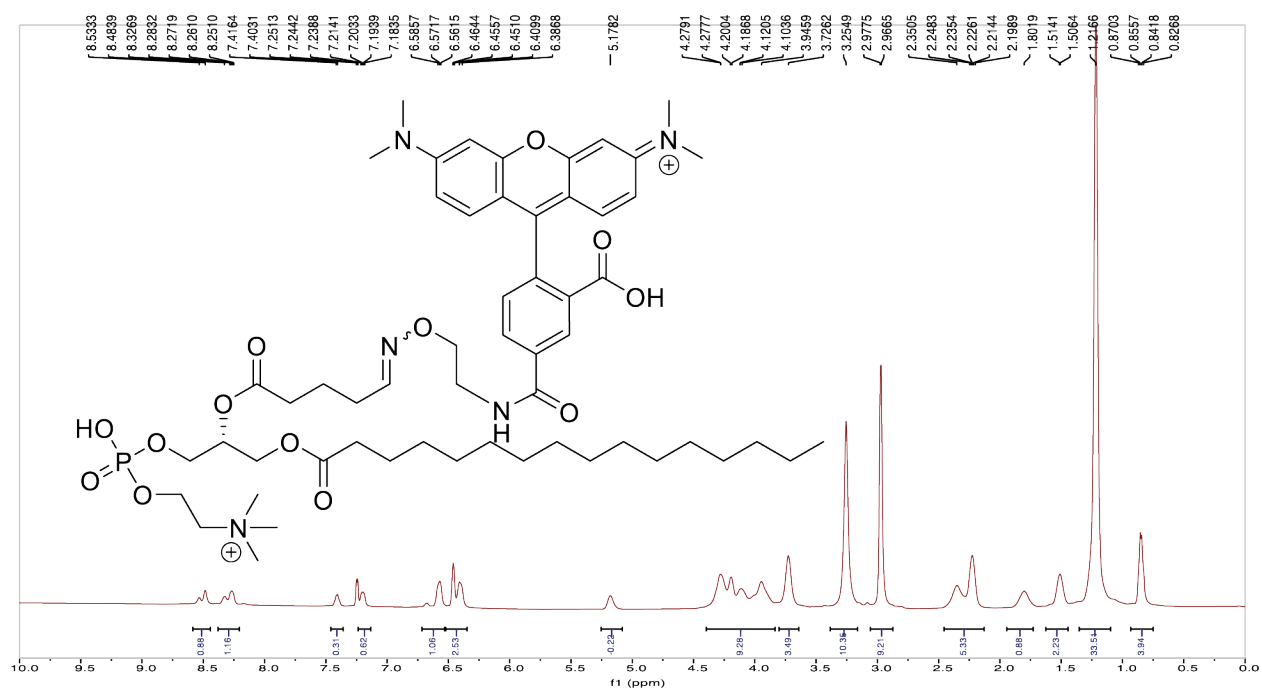


Figure S3.67. ¹H NMR spectrum of compound 9 (400 MHz, CDCl₃)

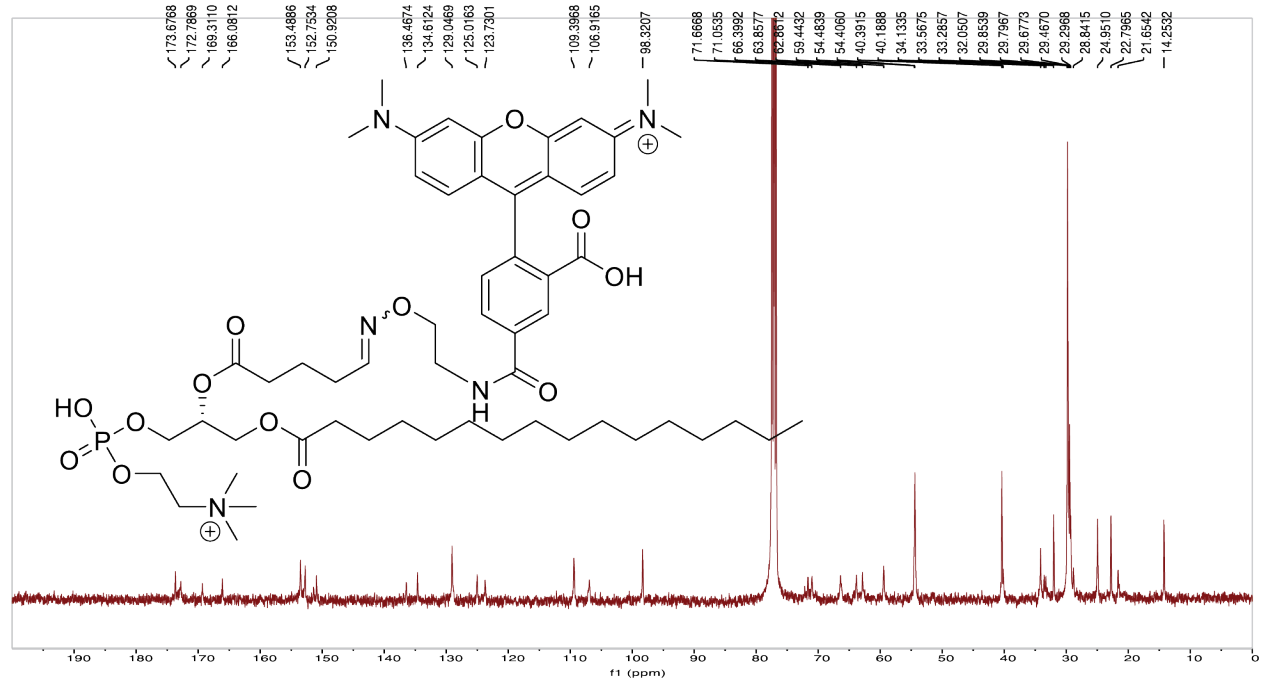


Figure S3.68. ^{13}C NMR spectrum of compound 9 (100 MHz, CDCl_3)

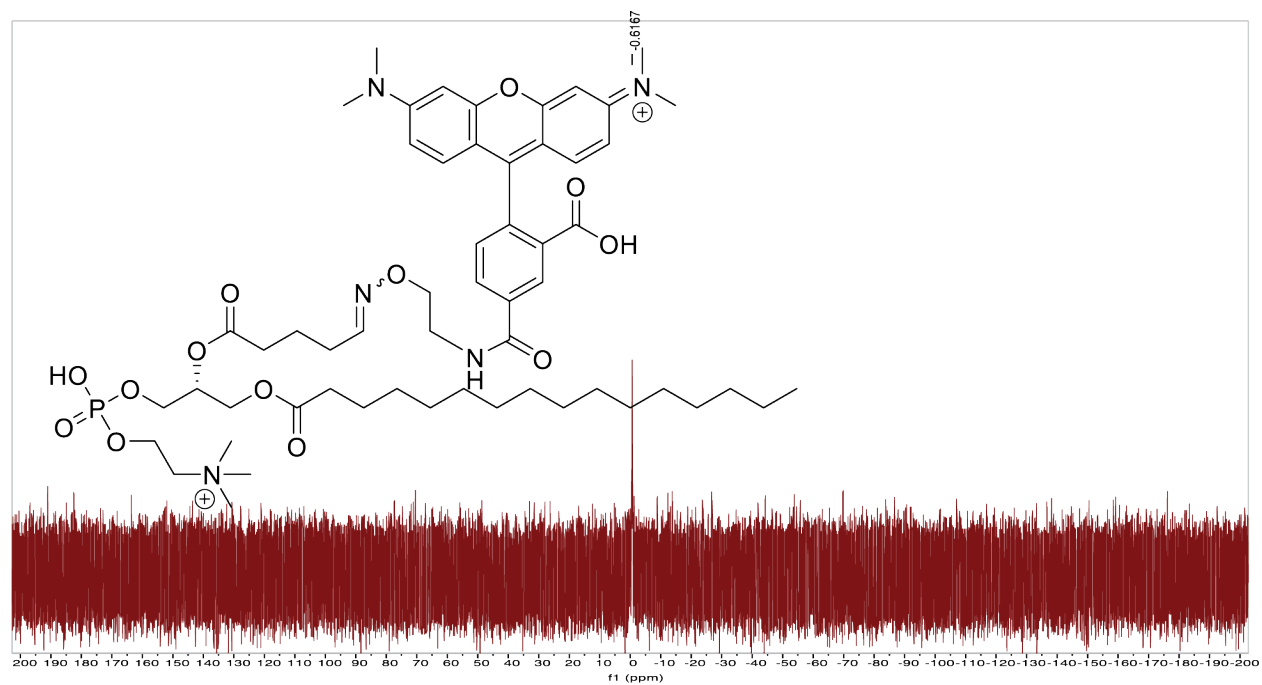


Figure S3.69. ^{31}P NMR spectrum of compound 9 (162 MHz, CDCl_3)

3.9.23 NMR spectra of compound 13

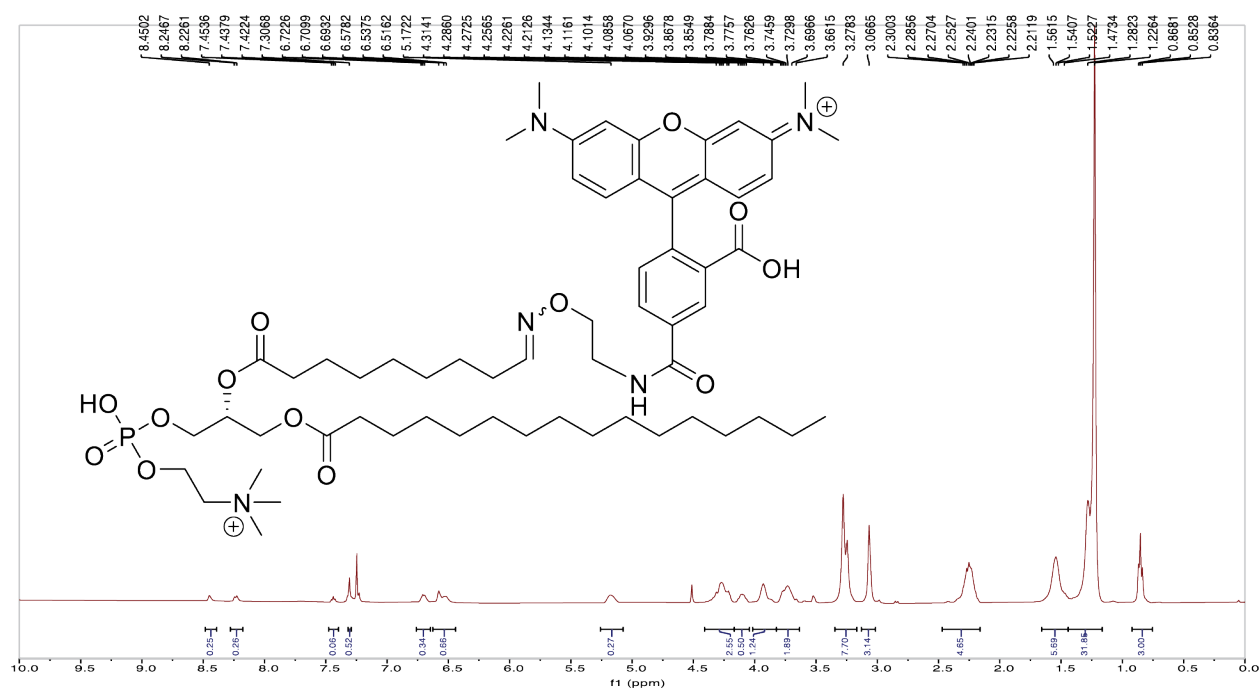


Figure S3.70. ^1H NMR spectrum of compound 13 (400 MHz, CDCl_3)

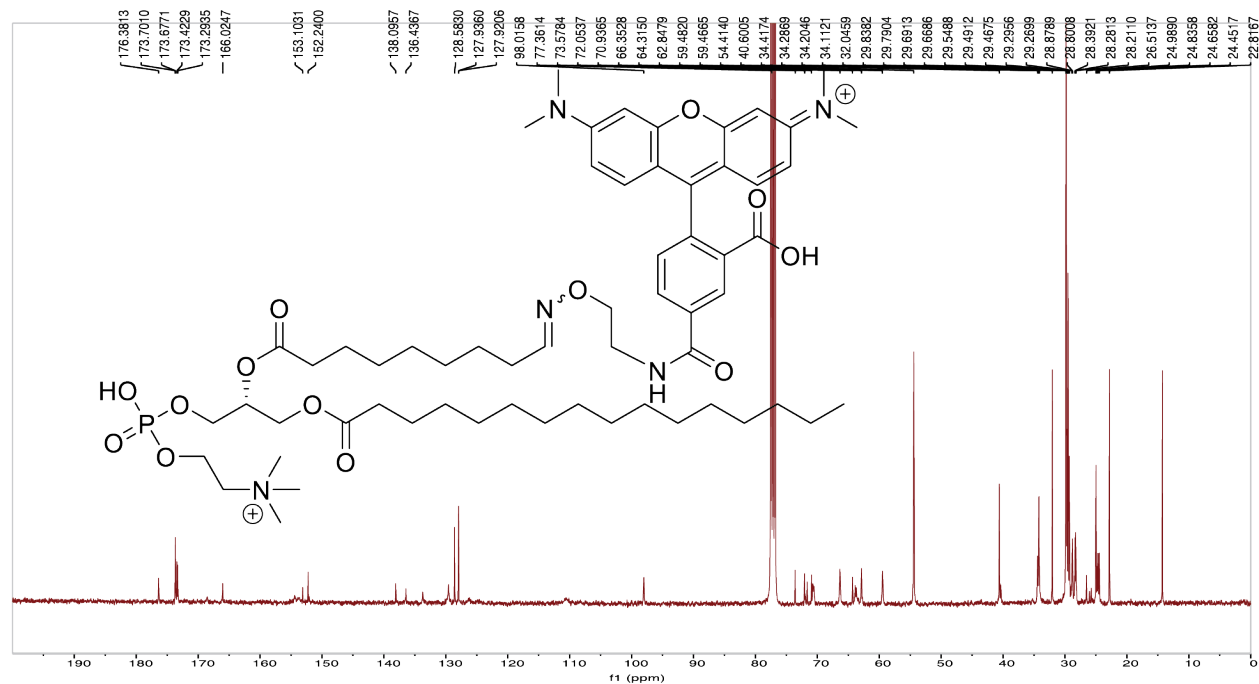


Figure 3.71. ^{13}C NMR spectrum of compound 13 (100 MHz, CDCl_3)

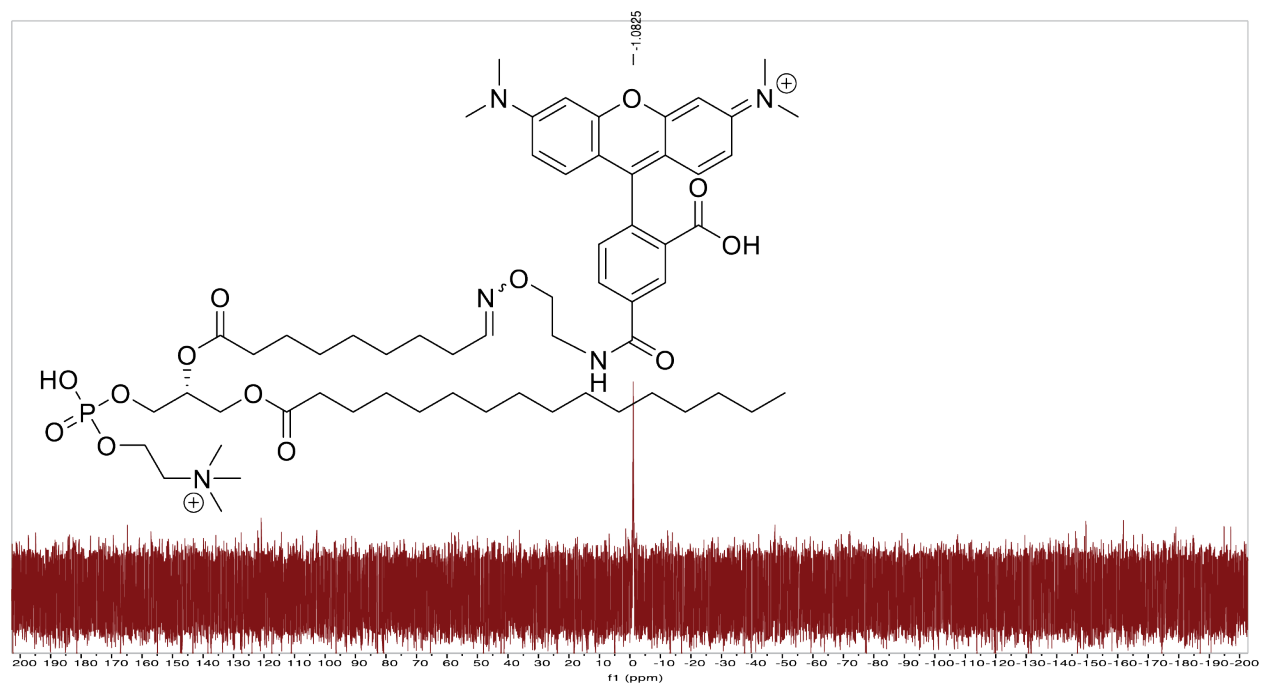


Figure S3.72. ^{31}P NMR spectrum of compound 13 (162 MHz, CDCl_3)

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