ACCOUNTS
of chemical research

Exploiting Bioorthogonal Chemistry to Elucidate Protein–Lipid Binding Interactions and Other Biological Roles of Phospholipids

MICHAEL D. BEST,* MENG M. ROWLAND, AND HEIDI E. BOSTIC
Department of Chemistry, the University of Tennessee, Knoxville, Tennessee 37996, United States

RECEIVED ON FEBRUARY 28, 2011

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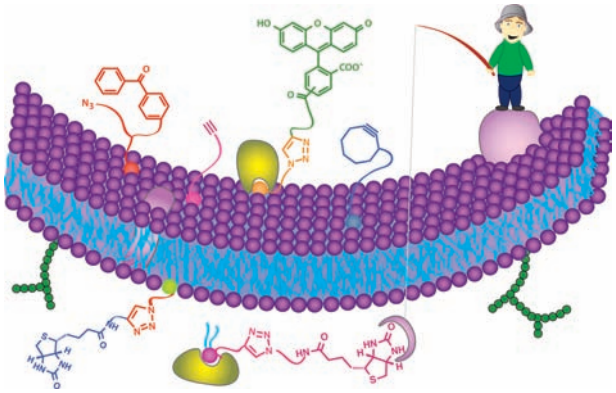


Lipids play critical roles in a litany of physiological and pathophysiological events, often through the regulation of protein function. These activities are generally difficult to characterize, however, because the membrane environment in which lipids operate is very complex. Moreover, lipids have a diverse range of biological functions, including the recruitment of proteins to membrane surfaces, actions as small-molecule ligands, and covalent protein modification through lipidation. Advancements in the development of bioorthogonal reactions have facilitated the study of lipid activities by providing the ability to selectively label probes bearing bioorthogonal tags within complex biological samples.

In this Account, we discuss recent efforts to harness the beneficial properties of bioorthogonal labeling strategies in elucidating lipid function. Initially, we summarize strategies for the design and synthesis of lipid probes bearing bioorthogonal tags. This discussion includes issues to be considered when deciding where to incorporate the tag, particularly the presentation within a membrane environment. We then present examples of the application of these probes to the study of lipid activities, with a particular emphasis on the elucidation of protein–lipid binding interactions. One such application involves the development of lipid and membrane microarray analysis as a high-throughput platform for characterizing protein-binding interactions. Here we discuss separate strategies for binding analysis involving the immobilization of either whole liposomes or simplified isolated lipid structures. In addition, we present the different strategies that have been used to derivatize membrane surfaces via bioorthogonal reactions, either by using this chemistry to produce functionalized lipid scaffolds that can be incorporated into membranes or through direct modification of intact membrane surfaces.

We then provide an overview of the development of lipid activity probes to label and identify proteins that bind to a particular lipid from complex biological samples. This process involves the strategy of activity-based proteomics, in which proteins are collectively labeled on the basis of function (in this case, ligand binding) rather than abundance. We summarize strategies for designing and applying lipid activity probes that allow for the selective labeling and characterization of protein targets. Additionally, we briefly comment on applications other than studying protein–lipid binding. These include the generation of new lipid structures with beneficial properties, labeling of tagged lipids in live cells for studies involving fluorescence imaging, elucidation of covalent protein lipidation, and identification of biosynthetic lipid intermediates. These applications illustrate the early phase of the promising field of applying bioorthogonal chemistry to the study of lipid function.

Published on the Web 05/06/2011 www.pubs.acs.org/accounts
10.1021/ar200060y

1. Introduction to Bioorthogonal Reactions and Lipid Signaling

Recent advancements in the development and optimization of bioorthogonal reactions have profoundly impacted applications in the field of chemical biology.[1,2] This chemistry allows for the selective labeling of a target biomolecule under ambient conditions within the complex environments of live cells and extracts, which has significantly advanced the ability to elucidate biological systems at the molecular level. Reactions including the copper-catalyzed azide–alkyne cycloaddition (CuAAC),[3–5] the copper-free variant exploiting strained cyclooctynes,[6–10] and the Staudinger ligation[11–17] possess numerous attributes that render them invaluable for biological applications. In particular, each involves reactive partners that undergo highly efficient coupling reactions under physiological conditions while avoiding side reactions with the litany of functional groups present in biological samples.

Lipids represent a crucial focus for current biomedical research due to the recent elucidation of their roles in regulating many vital biological pathways, and since aberrations in these signaling properties have been correlated to numerous debilitating diseases.[18–21] Furthermore, lipids are particularly interesting and challenging targets for studies due to the complex and diverse mechanisms by which they control biological processes. One prominent activity pertains to the actions of lipids as site-specific ligands in the binding of protein effectors. These binding events often drive the transient anchoring of soluble proteins onto the surfaces of cellular membranes, and in doing so regulate both the function and subcellular localization of the bound protein.[22,23] Diacylglcerol (DAG, Figure 1),[24–26] phosphatidic acid (PA),[26–28] and the family of phosphatidylinositol polyphosphates (PIP_ns)[29–31] are examples of lipids that interact with key proteins in this fashion.

DAG exhibits differential binding to the various isozymes that compose the protein kinase C (PKC) family, interactions that play key roles in regulating the cell life cycle and are aberrant in cancer.[18,19] PA has been determined to bind a group of protein effectors, such as Raf-1 kinase, again resulting in a key role in the regulation of cell proliferation.[32] However, the elucidation of PA activity is often difficult since protein targets generally lack a consensus binding sequence, and thus experimental approaches have been instrumental in identifying protein targets. In contrast to PA, the PIP_ns have been identified as the target ligands for multiple diverse families of binding modules containing conserved sequences that are present in a litany of protein effectors.[29–31] The complexities associated with both the numerous protein targets as well as the PIP_n family itself, consisting of seven isomers bearing different phosphorylation patterns on the *myo*-inositol headgroup, complicate the understanding of biological activities. A prominent PIP_n receptor is Akt, which contains a PH domain that binds phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), leading to the activation of this protein on the membrane surface through multiple phosphorylation events (Figure 2). Activated Akt stimulates cell growth, and thus, the enzymes that

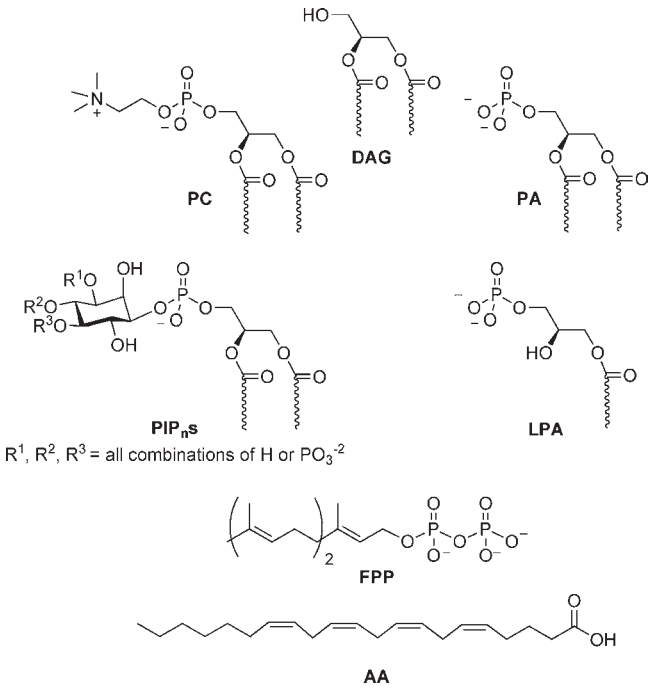


FIGURE 1. Examples of structures of biologically active lipids. Generic acyl chains are drawn due to the diversity of tail identities in nature.

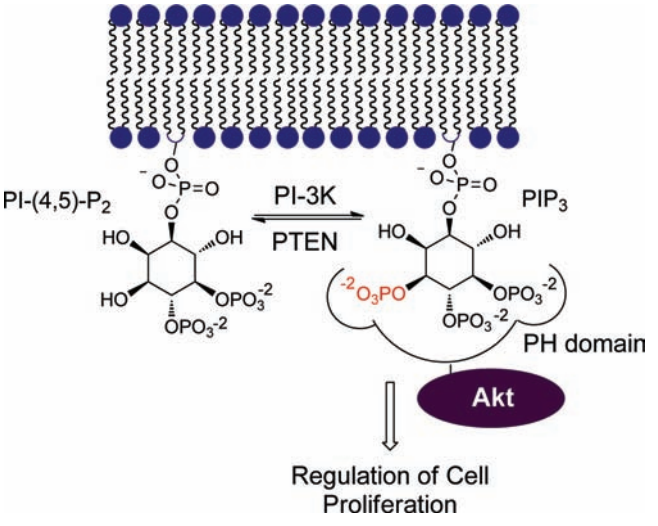


FIGURE 2. PIP_3, produced from phosphorylation of PI-(4,5)-P_2 by PI 3K, recruits Akt to the membrane, resulting in activation of cell growth.

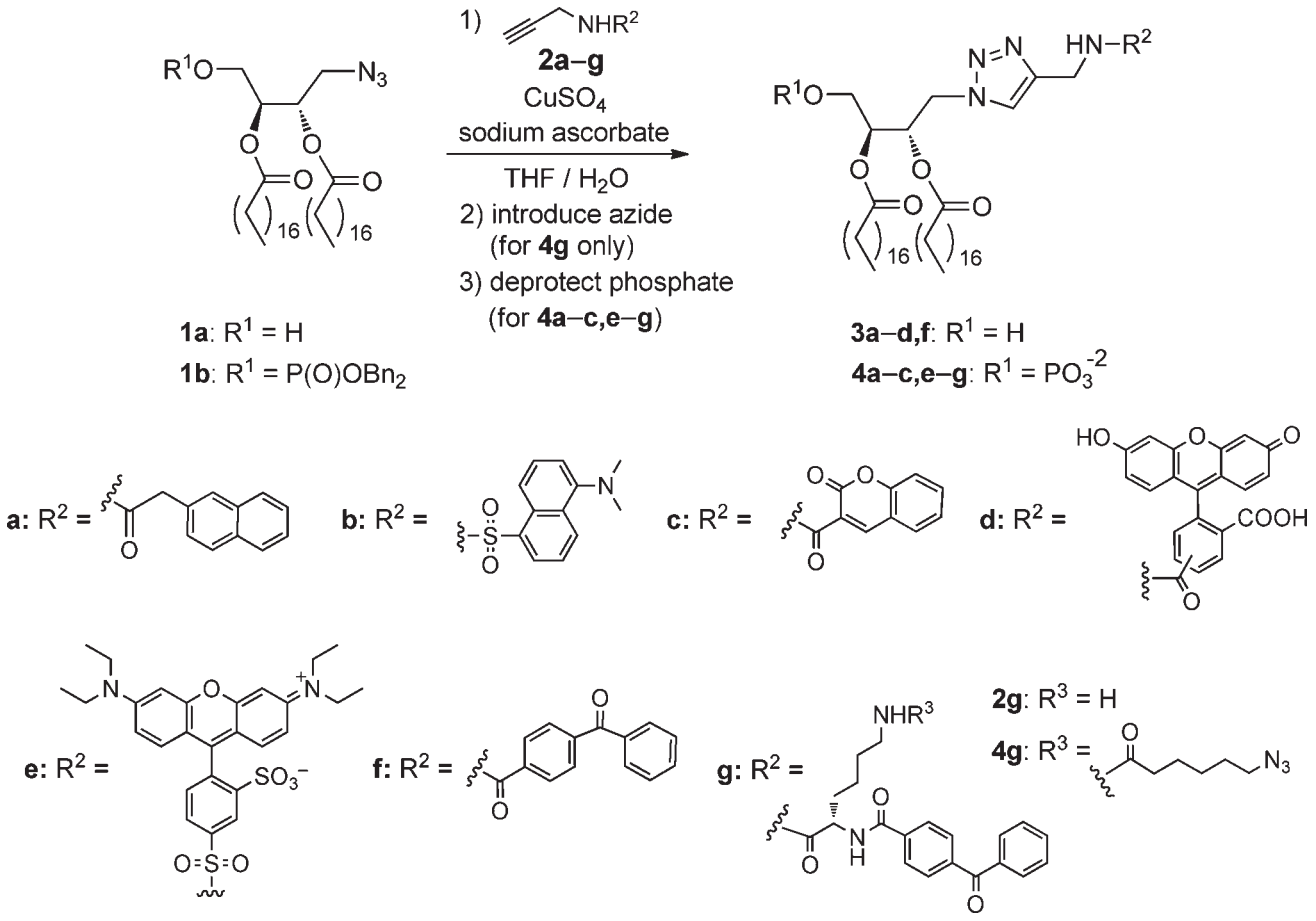


FIGURE 3. Synthesis of reporter-derivatized DAG and PA probes using modular scaffolds **1a** and **1b** containing azide groups at the lipid headgroup.

produce (phosphoinositide 3-kinase)[33] and modify (PTEN)[34] PIP_3 are among the most heavily mutated proteins in cancer.[21]

In addition to enforcing protein–membrane binding, lipids play other key roles as ligands and substrates that are more traditional of small organic molecules. An example is *lyso*-phosphatidic acid (LPA, Figure 1), which bears a single acyl chain and is produced from lipid precursors PA or phosphatidylcholine (PC). LPA binds a family of G-protein coupled receptor (GPCR) targets, the LPARs,[35] and exhibits growth-factor-like behavior that also goes awry in cancer. An additional example is arachidonic acid (AA), a fatty acid that is released via the hydrolysis of lipid acyl chains by phospholipase A2 (PLA2). AA acts as the biosynthetic entry into the prostaglandin pathway, which controls diverse processes including cell growth and pain responses.[36]

A third mechanism by which lipids play critical biological roles involves the attachment of lipids onto proteins.[37] Many proteins are modified and regulated via covalent lipidation in which either saturated (*S*-palmitoylation, *N*-myristoylation) or unsaturated (*S*-farnesylation with farnesyl pyrophosphate (FPP, Figure 1), *S*-geranylgeranylation) chains are appended onto amino acid residues. A prominent example of this is the Ras GTPase family, which is again commonly aberrant in cancer.[38] Ras proteins bearing a farnesyl moiety are present at the endoplasmic reticulum (ER) membrane, and the addition of one or more palmitoyl groups leads to cycling to the inner leaflet of the plasma membrane, where Ras activates cell growth. Covalent lipidation is an area in which bioorthogonal labeling strategies have had a profound effect. Although that area is beyond the scope of this Account, in which we focus primarily on noncovalent protein–lipid binding, covalent lipidation has been reviewed elsewhere.[39,40]

The complex and diverse mechanisms by which lipids control biological processes complicate the elucidation of their roles. As a result, a movement is now afoot to exploit bioorthogonal labeling strategies for applications aimed at characterizing lipid activities. Herein, we present a focused overview of the different approaches that we and others have pursued to harness the beneficial properties of bioorthogonal reactions to understand the biological roles

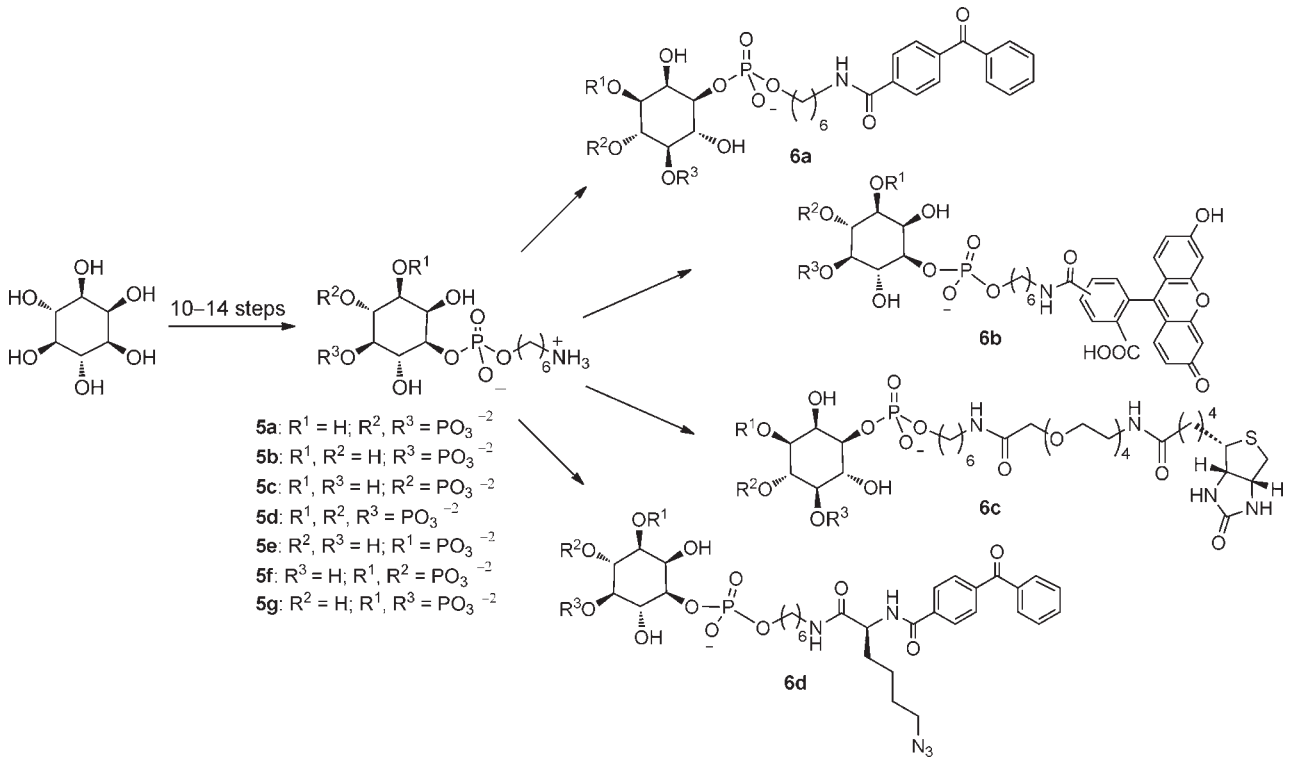


FIGURE 4. Synthesis of reporter-derivatized PIP_n headgroup analogues corresponding to all seven isomers.

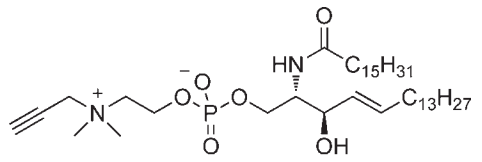
of lipids. We particularly focus on strategies aimed at characterizing protein–lipid binding interactions.

2. Design and Synthesis of Lipid Probes Bearing Bioorthogonal Tags

2.1. Introduction of Tags onto Lipid Headgroups. Due to the applications that can be achieved using bioorthogonal labeling, the design and synthesis of azide- and alkyne-tagged lipid probes has been a recent topic of substantial interest. In the derivatization of lipid structures, the location at which the reactive tag is introduced is important as this controls the presentation in the membrane. In certain applications, such as the labeling of proteins using photoaffinity tags,[41] it is beneficial to present reporter groups at the aqueous interface to enforce proximity to bound proteins. The tag placement also affects the accessibility for modification, in which presentation on the membrane surface may also be advantageous. Thus, we and others have been interested in generating synthetic lipid analogues bearing bioorthogonal labels at the headgroup.

Our initial efforts in producing lipid probes bearing bioorthogonal tags focused on azide-tagged DAG.[42] We envisioned the azide tag as being beneficial both for labeling experiments in complex samples and to generate a modular scaffold by which reporter groups could be conveniently introduced late in the synthesis to produce functionalized probes. Thus, we designed DAG analogue **1a** bearing an azidomethyl group in place of a hydrogen at the *sn*-1 headgroup position (Figure 3). Based on protein–membrane binding models, we hypothesized that introduction of reporter groups at this position would not greatly affect protein binding. Specifically, DAG-binding C1 domains consist of hydrophobic sequences that penetrate the membrane surrounding DAG, which we believed would tolerate reporter groups added at this particular position.

The synthesis of **1a** was complicated by significant *sn*-2 to *sn*-3 acyl chain migration in multiple routes that were pursued. This problem was circumvented by evaluating multiple protecting group strategies, eventually settling on the strategic use of a dimethoxytrityl group, culminating in an eight-step synthesis of **1a** from diethyl L-tartrate. CuAAC was then used to efficiently derivatize scaffold **1a** with alkynes **2a**–**d**,**f** to produce probes **3a**–**d**,**f**, including the addition of fluorescent (**3a**–**d**) and photoaffinity (**3f**) groups. Here, CuAAC was fortuitous as the mild conditions used for this reaction substantially limited byproducts arising from acyl migration, which was problematic when using other reactions for derivatization. We next studied the ability of these synthetic analogues to mimic the natural lipid in protein binding properties. Probes of type **3** were compared to unmodified DAG via incorporation into liposomes for a surface plasmon resonance (SPR) assay to evaluate PKC-binding. PKCδ showed virtually identical binding



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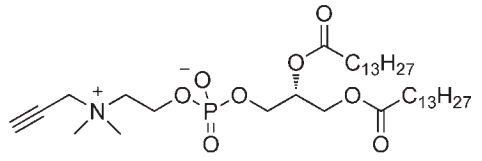

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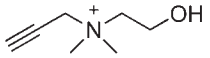

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FIGURE 5. Alkyne-tagged PC (**7**), SM (**8**), and choline (**9**) analogues.

to all analogues compared to natural DAG, demonstrating the validity of these compounds as probes.

Following the success of headgroup-modified DAG probes, we next extended this approach to develop probes corresponding to PA.[43] Due to previous complications in acyl migrations in synthesis, the route devised for modular azido-PA scaffold **1b** required installation of a protected phosphate and an azide moiety prior to the acyl chains. In this way, protected PA scaffold **1b** was synthesized in seven steps.[43] We then employed click chemistry to introduce reporter groups onto **1b** to produce **4a**–**c**,**e**–**g** as with before. Analogue **4g** is of note as a bifunctional activity probe containing both a benzophenone photoaffinity tag and an azide moiety as a secondary handle (see section 4). In this case, the binding properties of PA probes of type **4** were studied using an isolated PA binding domain, the C2 domain of PKCα, rather than a full length PKC, to increase the ligand binding specificity. While binding affinities fell off slightly compared to unmodified PA, the results were still comparable to unmodified PA standard.

The PIP_ns represent key targets for probe development due to their complex structures and prominent roles in key biological processes. However, the synthesis of azide-tagged PIP_n structures analogous to those of the aforementioned DAG and PA structures is complicated by the incompatibility of the azide group with certain phosphoramidite reagents[44] used to install PIP_n headgroups. Thus, as an alternate modular approach to PIP_n probes, we have used a tried and true strategy of amino-derivatized headgroup analogues[31] and have synthesized these modular intermediates corresponding to all seven of the naturally occurring PIP_n isomers (**5a**–**g**, Figure 4).[45] This effort benefitted from previously published procedures for PIP_n synthesis,[46–48] and it was again used to produce a number of PIP_n probes, exemplified by **6a**–**d**, for different studies. As illustrated by the production of **6d**, the azide group can be added after phosphodiester formation to circumvent the incompatibility of this group with certain phosphoramidite reagents.

In another effort, Cairo and co-workers have developed a modular synthetic route to PC (**7**, Figure 5) and sphingomyelin (**8**) analogues bearing an alkyne attached to the phosphocholine headgroup.[49] This strategy employs addition of a substituted propargylamine into a cyclic phosphotriester to introduce the alkyne tag. The resulting compounds were labeled by CuAAC in quantitative yields using a benzoxadiazole dye, and the sphingomyelin derivatives were shown to retain activity in sphingomyelinase assays.

Salic and co-workers exploited the incorporation of propargylcholine (**9**) into biosynthetic pathways to achieve the labeling of choline-containing phospholipids in live cells.[50] The resulting alkyne-containing phospholipids were labeled with fluorophores via CuAAC, and in subsequent imaging experiments the fluorescence signal was found to be localized to membranes and uniform across cells. Next, tandem mass spectrometry studies indicated that **9** was successfully incorporated into choline-containing phospholipids at normal levels and that the rest of the lipid population remained normal as well. It was also noted that **9** was incorporated more quickly into PCs (high levels after 24 h) than into the SM and ether phospholipid pathways. Finally, fluorescence and electron microscopy were used to image labeled phospholipids in mice, allowing the tracking of phospholipid synthesis.

2.2. Introduction of Tags into Lipid Acyl Chains. The development of lipids bearing bioorthogonal tags within the acyl chains has also been the subject of recent reports. In 2006, Smith and co-workers generated PC analogues bearing either alkyne (**10**) or azide groups at the terminus of the *sn*-2 acyl chain (Figure 6).[51] The efficient synthetic strategy employed the selective hydrolysis of the *sn*-2 acyl chain of PC using phospholipase A2, followed by coupling with either an azido- or alkynyl-fatty acid. CuAAC was then used to couple two of the resulting PC analogues to produce bolaamphiphiles of type **11**. These compounds did not form stable membranes, which was attributed to the polar nature of the triazole linkers. Additionally, Solaiman and co-workers have also exploited alkyne- and azide-tagged fatty acids, triacylglycerols, and glycolipids to chemically link different lipids, as exemplified by **12**.[52]

Smith and co-workers have also reported alkynyl-phosphatidylserine **13** and other analogues for imaging studies.[53] A

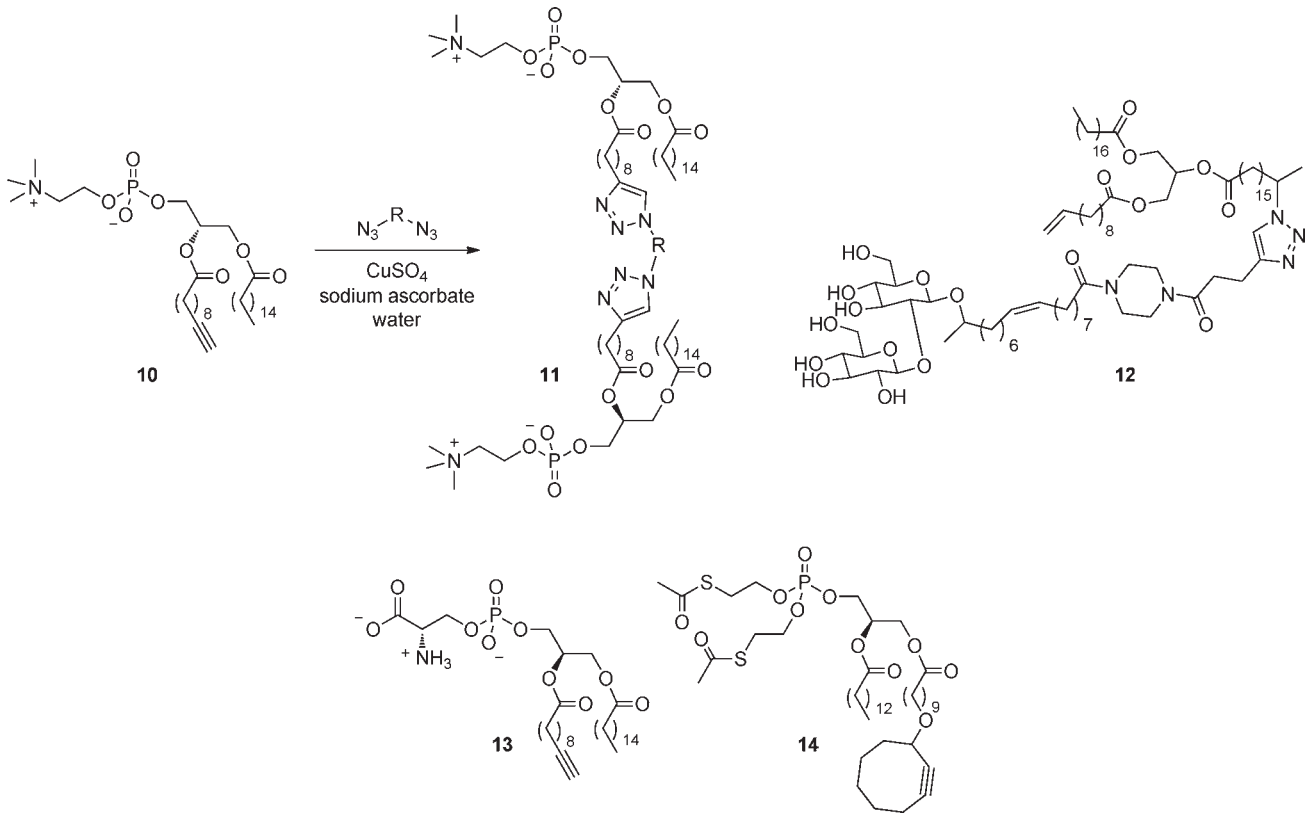

FIGURE 6. Lipid analogues bearing bioorthogonal tags within the acyl chains used for generating novel structures (**10**–**12**) and in vivo imaging (**13** and **14**).

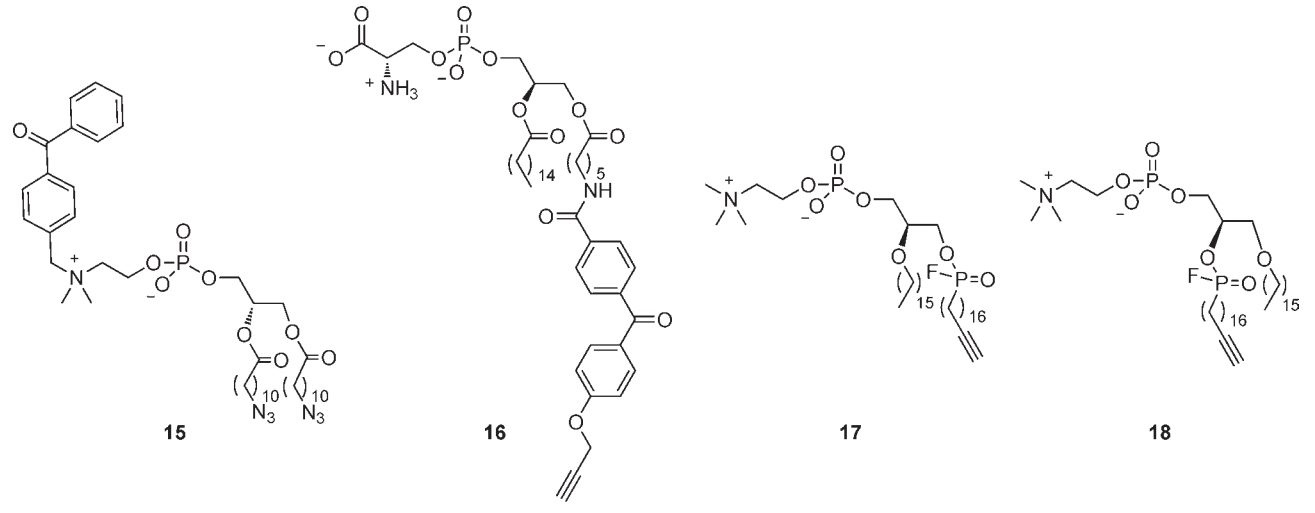

FIGURE 7. Lipid analogues bearing bioorthogonal tags within the acyl chains used for labeling lipid-binding proteins (**15** and **16**) and lipid-modifying enzymes (**17** and **18**).

chemoenzymatic approach was again employed, with phospholipase D used to hydrolyze the phosphodiester headgroup of a PC analogue, followed by coupling with a protected serine moiety and deprotection. Labeling of the resulting probes with fluorophores by CuAAC was then demonstrated. Schultz and Neef developed a series of alkyne- and cyclooctyne-labeled PA analogues including **14** that were applied for live cell fluorescence imaging experiments.[54] Cyclooctyne moieties were selected to avoid the need for copper during in vivo experiments. The phosphate moieties of the PA analogues were protected with *S*-acetylthioethyl groups to generate membrane permeable analogues that undergo subsequent

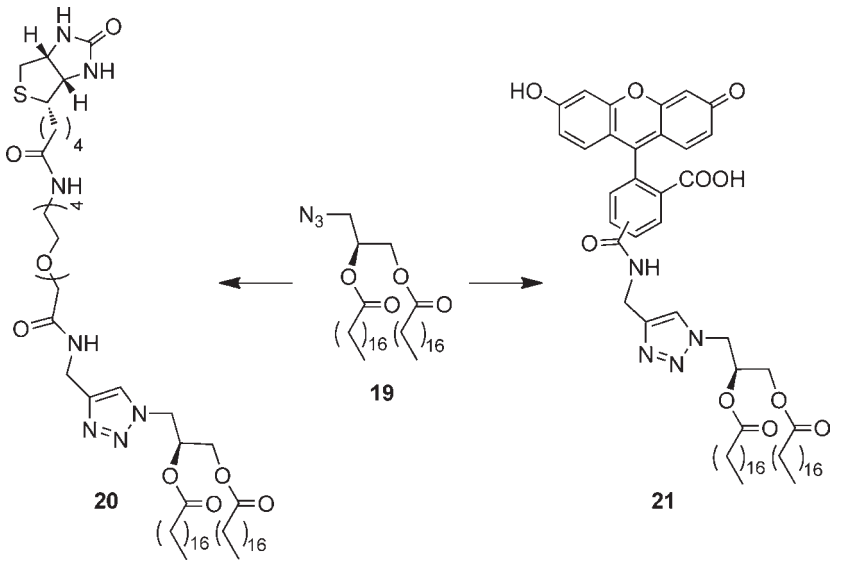

FIGURE 8. Derivatization of anchor **19** to produce lipids in order to decorate membrane surfaces.

enzymatic deprotection within the cell to release the desired PA analogue. Alkynes were introduced at the terminus of the *sn*-1 acyl chain by coupling alkynyl-fatty acids onto a protected glycerol intermediate. Fluorescence imaging of the PA analogues in live cells was then achieved through treatment with an azido-coumarin that is fluorogenic upon triazole formation.

To develop probes capable of labeling and identifying proteins that bind to PC, de Kroon, Liskamp and co-workers developed PC analogues of type **15** (Figure 7) bearing both a photoaffinity tag attached to the phosphodiester headgroup as well as azide tags at the termini of both acyl chains.[55] In the synthesis, one methyl group of the quaternary ammonium group of the precursor glycerophosphocholine was removed, followed by coupling of the resulting diol with two azido-fatty acids. The tertiary amine resulting from demethylation was then alkylated to introduce different photoaffinity tags in the final probes. The resulting compounds were exploited to characterize PC-binding receptors (see section 4). Bong and Bandyopadhyay have also reported bifunctional PS probes for use in characterizing lipid-binding proteins. In this case, the design incorporated both the benzophenone and alkyne tags within the same acyl chain, as shown in representative structure **16**.[56] Cravatt and Tully have developed probes **17** and **18** to identify and characterize lipid-modifying enzymes.[57] In this case, reactive fluorophosphonates were incorporated into either the *sn*-1 (**17**) or *sn*-2 (**18**) acyl chain to covalently label serine hydrolase enzymes that catalyze the hydrolysis of these substrates.

Long chain fatty acids and pyrophosphates bearing bioorthogonal tags have been exploited to infiltrate biosynthetic pathways and covalently label lipidated proteins, a topic which has been covered elsewhere.[39,40] These are commonly synthesized via the substitution of halogen or hydroxyl-containing precursors with azide, although syntheses involving prenyl groups are complicated by the production of multiple isomers upon displacement.[58]

3. Derivatization of Membrane Surfaces for Applications Including Microarray Analysis

Membrane systems such as liposomes and supported lipid bilayers (SLBs) are invaluable for diverse studies ranging from their use as model systems that mimic cellular membranes in the study of membrane processes[59−62] to applications pertaining to drug[63] and gene[64] delivery. Thus, bioorthogonal reactions have once again found application for such endeavors. For example, due to the complex nature of protein−membrane binding interactions, we have been interested in developing microarray analysis as a high-throughput platform for characterizing the details of these recognition events.

To develop a high-throughput assay for membrane binding, we first set out to perform the immobilization of liposomes to achieve surface-based detection of binding.[65] Here, we again exploited CuAAC, in this case by developing azido-lipid anchor **19** as a modular scaffold for introducing functionality onto the membrane surface (Figure 8). For example, biotin-lipid conjugate **20** was developed to generate liposomes that could be loaded onto streptavidin-coated surfaces, and fluorescent derivatives such as **21** were devised for optical detection during studies. In this system, the triazole resulting from CuAAC was envisaged to be beneficial due to its polarity, causing it to prefer presentation

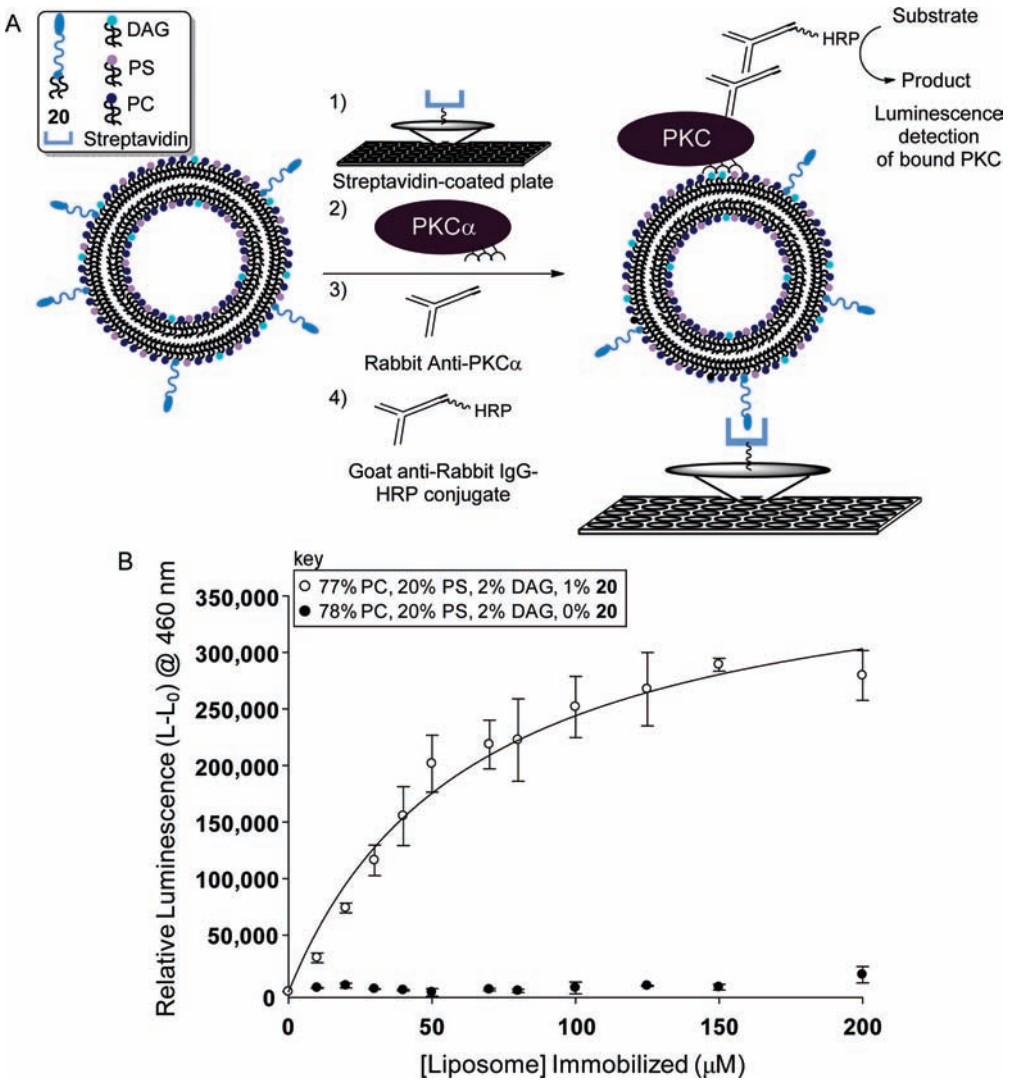

FIGURE 9. Microplate assay for protein–membrane binding. (A) Schematic depicting the binding assay. (B) Representative binding isotherm for protein kinase C association.

at the aqueous interface, as had been described by Smith and co-workers.[51] Furthermore, we have found these triazole-linked compounds to be easier to purify and characterize than traditional phospholipids.

We next moved to apply these compounds to study liposome immobilization. Since membranes are notorious for undergoing nonspecific processes including surface anchoring, fusion, and unraveling, initial control studies were necessary. First, fluorescence-based studies involving the immobilization of liposomes tagged with **21** both containing and lacking **20** demonstrated that the biotin–lipid anchor is necessary for surface attachment. In addition, dye leakage assays using fluorescein-encapsulated liposomes indicated that liposomes remained intact upon immobilization and were only cleaved following detergent addition, detected via dye activation upon release.

Following successful characterization of liposome immobilization, a microplate-based protein–membrane binding assay was achieved. Initially, we analyzed the binding of PKCα to immobilized liposomes composed of PC, PS, DAG, and **20**. The detection assay involved liposome immobilization followed by incubation with PKCα and chemiluminescence-based detection of bound protein via ELISA (Figure 9A). The resulting binding isotherms (Figure 9B) indicated sensitive detection of protein binding to immobilized liposomes with little-to-no background observed when the assay was run with liposomes lacking **20**. This approach provides an efficient method for characterizing the binding of proteins to membrane bilayers.

In our approach to liposome derivatization, we chose to exploit CuAAC as a synthetic transformation to introduce functionality onto lipid scaffolds prior to liposome formation.

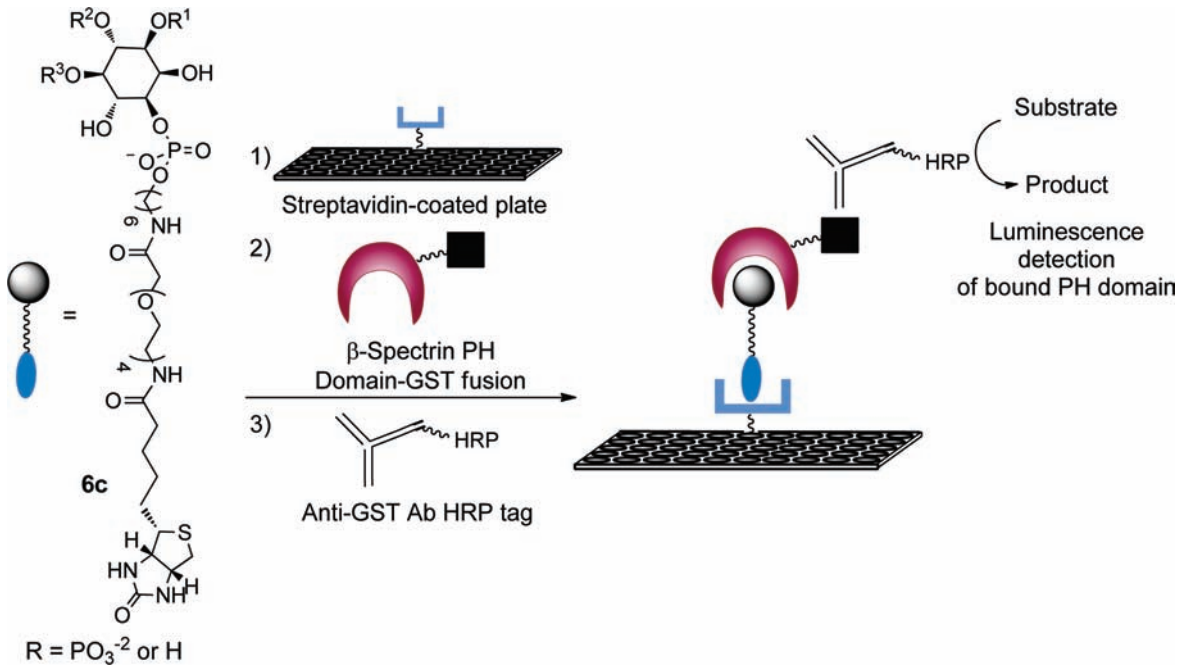

FIGURE 10. Microplate binding assay for protein–PIP_n headgroup interactions.

However, other groups have shown that the decoration of intact liposomes is also effective using CuAAC. In 2006, Kros and co-workers demonstrated CuAAC on liposomal surfaces using FRET to characterize the reaction of an azide-tagged FRET donor with an alkyne-lipid embedded within a liposome that was also labeled with a FRET acceptor.[66] Concurrently, Schuber and co-workers used CuAAC to introduce azide-containing sugars onto liposomes bearing alkyne groups.[67] Subsequent binding studies with concanavalin A lectin indicated that the sugars were accessible for binding on the resulting membranes. Sun and co-workers have also introduced carbohydrates onto intact liposomes through the Staudinger ligation.[68] Boxer and co-workers used CuAAC to tether azide-containing liposomes onto SLBs decorated with alkynes in order to characterize binding interactions mediated by oligonucleotides also present on the liposomal surfaces.[69] Finally, CuAAC has also been exploited to derivatize the surfaces of polymerized liposomes, which benefit from their enhanced stability.[70] This chemistry was then used to introduce both fluorescent and RGD peptide tags for cell imaging experiments.

In addition to our microplate assay employing whole liposomes, we have also pursued assays involving the immobilization of isolated lipid components due to the diverse binding modes of lipid-binding proteins. Specifically, while certain proteins require the membrane for binding, generally because the protein inserts hydrophobic regions into the bilayer, other proteins bind lipid headgroups with high affinity in the absence of the membrane environment.[29,30] Thus, we also sought a rapid assay for protein binding employing synthetic PIP_n headgroup motifs as a high-throughput platform for characterizing protein–PIP_n binding interactions.[71]

Due to the incompatibility of azide groups with certain phosphoramidite reagents used for PIP_n headgroup synthesis, we exploited PIP_n analogues derived from headgroup amino-conjugates, as previously described in Figure 4. In the assay, synthetic biotin-PIP_n headgroup conjugates of type **6c** were immobilized onto streptavidin-coated 96-well microplates and then subjected to surface-based binding analysis using ELISA detection (Figure 10). We first used this approach to analyze the binding of the PH domain of β-spectrin to PI-(4,5)-P_2. The strong dissociation constant measured for this binding event (low nM K_d) confirmed that this PH domain binds the PIP_n headgroup with high affinity outside of the membrane. Control ligands were used to rule out nonspecific ligand or protein binding during studies. This assay was also effective for studying inhibition of receptor–PIP_n binding via competition experiments, which is of interest for identifying inhibitors with therapeutic potential.

4. Probe-Based Approaches for Identifying Lipid-Binding and Lipidated Proteins

Another challenging aspect of lipid signaling is the identification of proteins that bind lipids, an endeavor in which bioorthogonal reactions have again proven fortuitous due

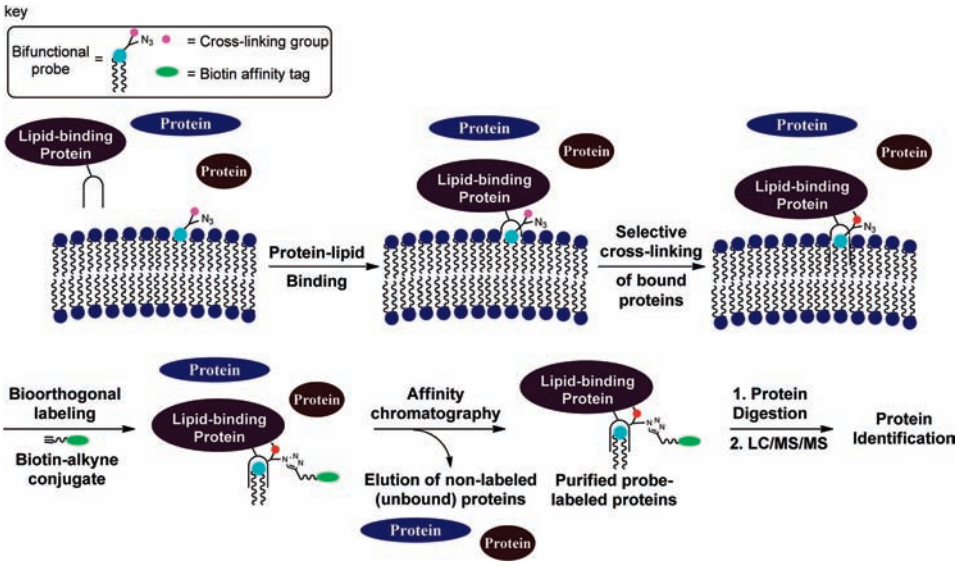

FIGURE 11. Discovery of lipid-binding proteins using bifunctional probes. Probes are first incubated with a cell lysate or live cell. Covalent labeling of probe-bound proteins is then enforced via irradiation of the affinity tag. Probe-labeled proteins are next selectively purified or detected via the secondary tag, followed by analysis of resulting labeled proteins using digestion and tandem mass spectrometry.

to the ability to selectively purify and identify proteins after labeling with probes bearing bioorthogonal tags. This is the basis for the strategy of activity-based protein profiling (ABPP), which entails the collective characterization of proteins based on function rather than abundance.[72] Activity probes generally consist of synthetic substrate or ligand analogues in which two groups are introduced: (1) a group that allows selective −covalent labeling of target proteins following binding, for which a photoaffinity tag[41] is commonly used for ligands such as lipids; and (2) a secondary handle, often a bioorthogonal tag, that is derivatized to achieve the purification or detection of the resulting protein−probe adducts from a complex mixture. Bioorthogonal chemistry is typically used to introduce either a fluorescent dye for on-gel imaging or a biotin for purification via affinity chromatography. The probe is then employed to label, purify, and detect proteins from cell extracts, live cells, or whole organisms, as depicted in Figure 11. While this illustration depicts the capture of peripheral proteins, the lipid activity probe strategy is also effective for labeling integral membrane proteins that bind lipids. This approach complements other current methods for lipid analysis, including the use of GFP-tagged protein reporters as well as mass spectrometry, IR, and Raman based imaging methods.[73]

In an initial demonstration of this strategy, de Kroon and co-workers utilized PC probes of type **15** (Figure 7) to label proteins that interact with this lipid.[55,74] These probes contained one of a series photoaffinity tags (benzophenone, phenyl azide, or diazirine) linked to the phosphodiester moiety of the PC headgroup as well as azides at the termini of both acyl chains. In initial in vitro experiments, large unilamellar vesicles containing these probes were found to successfully cross-link to apo-cytochrome c, as evidenced by enhanced carbonate wash resistance. Next, these compounds were employed for labeling experiments to identify protein targets in the mitochondrial membranes of *Saccharomyces cerevisiae*. Following photo-cross-linking, CuAAC was exploited to achieve on-gel fluorescence imaging and protein identification through biotin labeling, affinity chromatography, digestion, and mass spectrometry based proteomics. Probes bearing different photoaffinity tags were found to label different although overlapping groups of proteins, suggesting that the use of multiple probes containing various tags is beneficial. This study culminated in the detection of multiple proteins, including proteins previously known to bind PC as well as some newly discovered protein targets. In addition to lipid-binding proteins, activity-based probes can also be used to label lipid-modifying enzymes, as was recently demonstrated by Cravatt and Tully through the characterization of phospholipases using probes **17** and **18** (Figure 7).[57]

This lipid activity probe strategy presents an exciting and efficient approach to the identification and characterization of lipid-binding proteins. Our group has been performing similar studies to identify peripheral proteins that target key signaling lipids. We have designed and synthesized

bifunctional probes corresponding to lipids including PA (**4g**, Figure 3) and the PIP_ns (**6d**, Figure 4), as described in section 2.1. These compounds have been shown to retain their protein binding properties despite the addition of tags at the lipid headgroups. In initial in vitro labeling experiments, these probes have been found to successfully label known proteins, and controls involving either small molecule competition or protein denaturation have yielded abrogation. Finally, these compounds led to the labeling of numerous proteins in cell extracts, results that will be reported in due course.

Otherwise, Brown, Porter, and co-workers have also used synthetic alkyne-tagged lipids to identify metabolic products through capture and release via cobalt complexes and detection via mass spectrometry.[75] In addition, bioorthogonal labeling has been highly useful for characterizing covalent lipidation of proteins, which has been reviewed elsewhere.[39,40] Aberrant covalent protein modifications have also been studied in this way. For example, Marnett and co-workers identified proteins labeled by 4-hydroxynonenal, which results from lipid peroxidation, using analogues bearing bioorthogonal tags.[76−78]

5. Conclusions

Lipid signaling events comprise a complex and diverse series of regulatory processes that present numerous challenges for elucidating details at the molecular level. As such, recent advancements in bioorthogonal chemistry show great prospects for producing a paradigm shift in the approaches used to characterize lipid activity. The examples discussed herein depict a promising start for such studies, but much work remains due to the broad scope of lipid-driven events that must be elucidated. While advancements in synthesis have allowed the generation of tagged analogues of highly complex lipids, such as lipid A,[79] the diversity of biologically active lipid structures will keep us busy for some time. Otherwise, there are aspects of this chemistry that could use improvement. For example, a primary concern when deploying probes in living cells or organisms is that these compounds undergo subsqequent enzymatic modification. This can be a benefit when studying or identifying metabolic downstream targets, but it is problematic when the compound being studied undergoes transformations. Thus, the further advancement of the methods by which the structural integrity of probes is maintained within biological contexts, such as the use of metabolically stabilized analogues,[31] would be beneficial. In this way, enhancing the technology that complements bioorthogonal reactivity will allow the full potential of this chemistry to be realized.

BIOGRAPHICAL INFORMATION

Michael Best received his B.S. in Chemistry from Boston College, where he pursued undergraduate research with Lawrence T. Scott. He next performed his doctoral work with Eric V. Anslyn at the University of Texas at Austin, followed by a postdoctoral stint at The Scripps Research Institute in La Jolla working with Chi-Huey Wong. In 2005, he began his independent career at the University of Tennessee at Knoxville, where a primary focus of his group's research has been the design, synthesis, and application of lipid probes to study the biological activities of phospholipids.

Meng Rowland was born in Changchun city in China. In 2004, she obtained her B.S. degree from Jilin University in Changchun with a major in pharmaceutical sciences, and 2 years later earned her M.S. degree in medicinal chemistry. In August 2006, she joined the Chemistry Department at the University of Tennessee to pursue her doctoral research. There, she has worked with Dr. Michael Best on the synthesis and application of lipid probes to study biological activities.

Heidi E. Bostic was born in Gaffney, South Carolina. In 2003, she began her undergraduate studies at Wofford College in Spartanburg, SC, where she earned a B.S. in Chemistry and a B.A. in Computer Science. Upon graduating in Summer 2007, she began her career as a graduate student at the University of Tennessee. She then joined the lab of Dr. Michael Best, and her research interests have included the application of bioorthogonal chemistry to study membrane systems.

This Account was made possible by funding from the National Science Foundation (CHE-0954297 and DMR-0906752) and the University of Tennessee. We are extremely thankful to Carolyn Bertozzi for providing us with the opportunity to contribute to this exciting special issue. In addition, we are thankful to the many colleagues who have assisted with certain studies described in this manuscript through collaborations and helpful discussions, including Rob Stahelin, Wonhwa Cho, Glenn Prestwich, Ben Cravatt III, Brad Smith, and Ned Porter. We also thank Hollie B. Pennington for assistance with graphic design.

FOOTNOTES

*To whom correspondence should be addressed. Telephone: (865)-974-8658. Fax: 865-974-9332. E-mail: mdbest@utk.edu.

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