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The collage consists of six panels arranged in a 3x2 grid:

- Top Left:** A schematic diagram of a laboratory-scale reactor system for the conversion of aromatic carboxylic acids (e.g., HOOC-C₆H₄-COOH) into various products. The system includes a reactor vessel, condenser, and analytical equipment. A photograph of a white crystalline product is shown. **p 7421**
- Top Right:** A 3D molecular model illustrating the metabolism of fatty acids. It shows the conversion of a Fatty Acid (e.g., $\text{CH}_3(\text{CH}_2)_n\text{COOH}$) into Fatty-CoA, Fatty Ethanolamine, Fatty Amide, Fatty Glycine, and Fatty Amide via intermediates like GLYAT, Cytochrome c, BAAT, and ADH. **p 7343**
- Middle Left:** A schematic of a photolabeling experiment. It shows a membrane with proteins (green ovals) being exposed to light (represented by a lightbulb icon). The process is labeled "photolabeling". **p 7880**
- Middle Right:** A schematic diagram of a membrane separation process. A cylindrical membrane is shown with "Feed" entering from the left and "Permeate" exiting at the bottom, while "Retentate" is collected above. **p 7728**
- Bottom Left:** Two images. The left image shows a red bridge-like structure labeled "Borides" and "Phosphides" against a blue sky. The right image shows a dark, fibrous material labeled "Bulk". **p 7981**
- Bottom Right:** A complex molecular diagram showing the interconversion of various fullerenes (C₆₀, C₇₀, C₇₆, C₇₈, C₈₀) and their derivatives, some substituted with methyl (Me) and bromine (Br) groups. **p PR179-PR233**



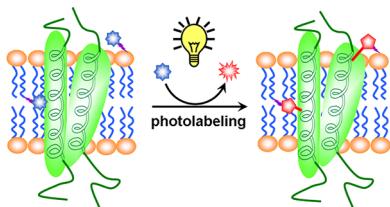
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Photoactivatable Lipid Probes for Studying Biomembranes by Photoaffinity Labeling

Yi Xia and Ling Peng*

Aix-Marseille Université, Centre Interdisciplinaire de Nanoscience de Marseille, CNRS UMR 7325, Campus de Luminy, 13288 Marseille, France



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1. INTRODUCTION

1.1. Biomembranes

Biological membranes consist mainly of lipids and proteins (Figure 1) and form the boundaries of all cells. The major lipids

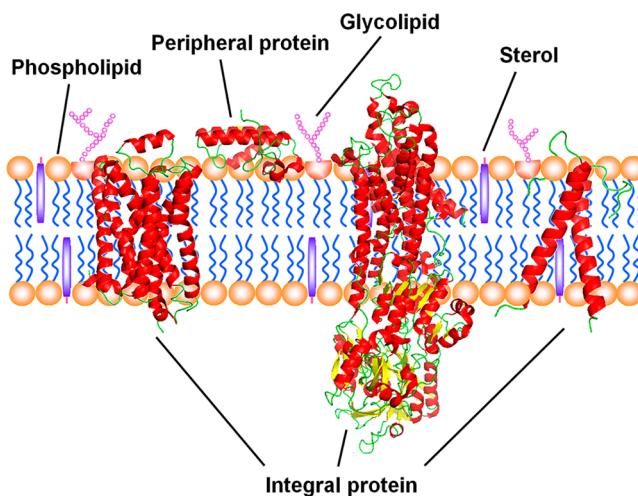


Figure 1. The fluid mosaic model of biological membrane proposed by Singer and Nicolson.¹ Lipid molecules form the membrane bilayer with membrane proteins inserted or associated into the bilayer. The major lipids in biomembranes include phospholipids, glycolipids, and sterols. The membrane proteins can be divided into peripheral proteins and integral proteins. The integral proteins from left to right are the β 1-adrenergic receptor,² rabbit skeletal muscle Ca^{2+} -ATPase,³ and glycophorin A.⁴ The peripheral protein is human group X secreted phospholipase A₂.⁵

present in biomembranes are phospholipids, which share a common feature with glycolipids and sterols (Figure 2) in that they bear both hydrophilic and hydrophobic moieties and act as amphiphilic molecules. Because of the hydrophobic interactions among their fatty acyl chains and the interaction of their hydrophilic heads with aqueous media, phospholipids and glycolipids can self-organize spontaneously as a bilayer into which sterol molecules can be inserted (Figure 1). Proteins associated with such biomembranes are classed as either

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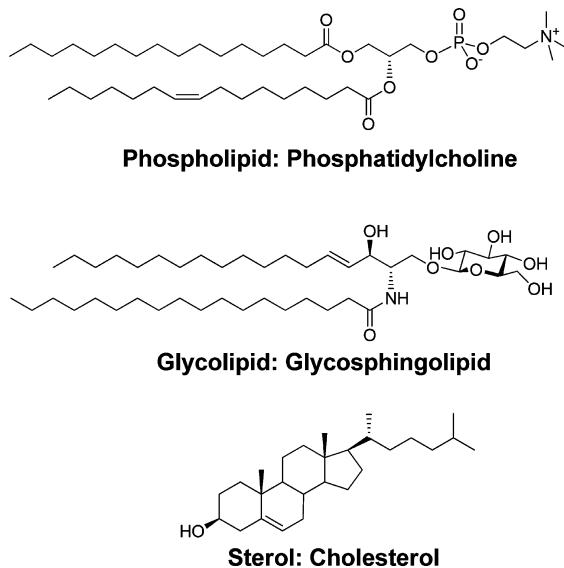


Figure 2. Representative structures of a phospholipid, a glycolipid, and a sterol.

peripheral or integral (Figure 1), the integral ones being either partly inserted into or spanning across the lipid bilayer, and the peripheral ones usually being located at the membrane surface via interactions with the polar part of lipids and/or direct binding to integral proteins.

Our understanding of the biological membrane began with the fluid mosaic model proposed by Singer and Nicolson in 1972 (Figure 1).¹ In this model, diverse lipids and proteins were believed to be confined within the membrane bilayer wherein they exhibit free lateral diffusion. In mammalian cell membranes, the chain length and degree of unsaturation of the fatty acids, as well as the cholesterol content, greatly affect the membrane fluidity. Mounting evidence, including that of inner- and outer-leaflets containing different lipids, favors a non-random architecture of the plasma membrane with lateral segregation of the lipid bilayer producing unique lipid-ordered microdomains, the so-called “lipid rafts” (Figure 3).^{6,7} These rafts refer to regions of high concentration of sterols (cholesterol in mammals) and sphingolipids (sphingomyelin and glycosphingolipids) associated with various signaling and transport proteins.

Over the past 40 years, despite much research effort dedicated to studying the structure and properties of biomembranes,^{11–17} our understanding remains limited and is far from complete. What is clear is that, in addition to serving as a barrier to protect the cells they surround, biological membranes are also involved in many essential biological processes, such as signal transduction, energy conversion, transport of ions and molecules across the membrane, etc.^{18,19} Moreover, membrane proteins are of particular importance in drug discovery, with over 60% of the drugs currently on the market being targeted to membrane proteins.²⁰ Among the various membrane proteins, G protein-coupled receptors (GPCRs) and ion channels are considered as the two major drug targets because of their extremely crucial roles in maintaining cellular function.^{21–25} In 2003, the Nobel Prize in Chemistry was awarded to Peter Agre and Roderick MacKinnon for their contribution to the discovery of functionally relevant channels in cell membranes. Robert Lefkowitz and Brian Kobilka then won the Nobel Prize in

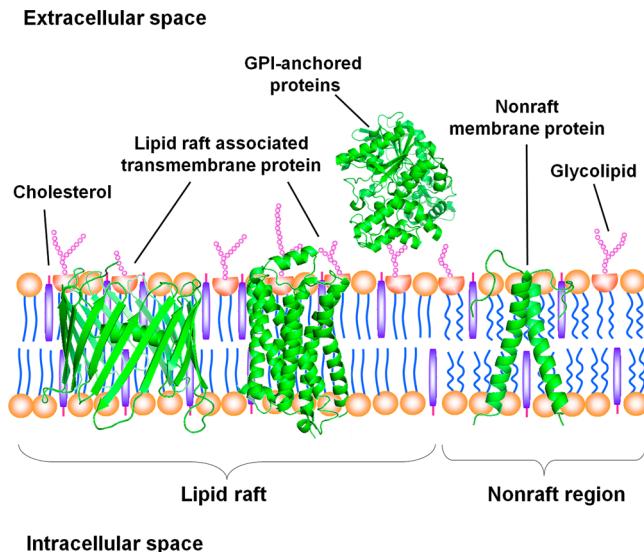


Figure 3. Schematic representation of a lipid raft.^{6,8} Rafts are liquid-ordered domains that are more tightly packed than the surrounding nonraft region of the bilayer. They group together a subset of transmembrane or glycosylphosphatidylinositol (GPI)-anchored proteins with lipids rich in cholesterol and sphingolipids. The lipid raft associated transmembrane proteins from left to right are human voltage-dependent anion channel 1 (VDAC1)⁹ and β 1-adrenergic receptor.² The GPI-anchored protein is acetylcholinesterase.¹⁰ The nonraft membrane protein is glycophorin A.⁴

2012 for tracking down GPCRs and revealing the atomic details of how these proteins work,^{26,27} highlighting again the significance of membrane proteins in chemistry and related research fields. Studying the structure and function of membrane proteins and analyzing their protein–lipid interactions is therefore of paramount importance to enhance our understanding of biomembranes and allow the identification and investigation of membrane proteins as therapeutic targets for future drug development programs.

1.2. Photoaffinity Labeling of Biomembranes

Obtaining structural information on biomembranes with methods such as X-ray crystallography and NMR analysis has been rendered difficult due to their complex heterogeneity and insolubility in water.^{14,15,28} The pioneering study by Khorana in 1975 using photoaffinity labeling²⁹ provided a direct method of investigating biomembranes at the molecular level to identify the membrane proteins and study the sites of lipid–protein interaction.^{30–39} Highly competent photoactivatable lipid probes are the prerequisite in this classic but very useful method (Figure 4). The lipid probes bear photoactivatable chromophores either at the polar head or within the hydrophobic part, and are able to incorporate into the lipid bilayer. When exposed to light, they generate highly reactive species allowing their covalent cross-linkage with membrane proteins or surrounding lipids. Lipids that interact with the photoactivatable lipids⁴⁰ can be directly identified, while further proteolysis and sequencing of the labeled proteins enables structural analysis of the lipid–protein interaction sites, thus supplying valuable structural and functional information on biomembranes (Figure 4).

So far, both lipid and nonlipid probes have been used in photolabeling studies of biomembranes. The use of the nonlipid probes (usually small, hydrophobic molecules) was extensively reported in the early years. For example, the small

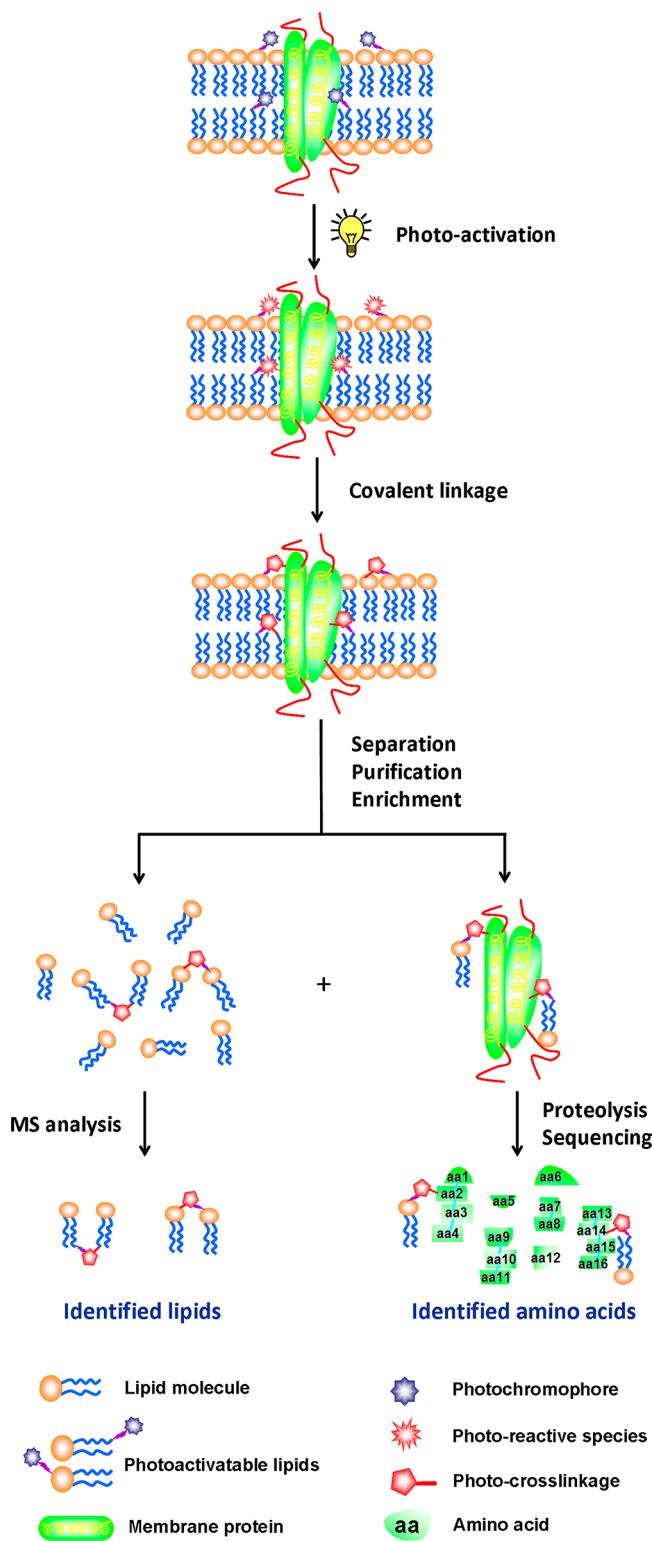


Figure 4. Schematic presentation of photoaffinity labeling study of the biomembrane using photoactivatable lipid probes. The photoactivatable lipids are first incorporated into the biomembranes. Upon irradiation, they generate highly reactive species and form covalent cross-linkages with their target proteins or surrounding lipids. Lipids covalently labeled by the photoactivatable lipids can be also identified by lipid analysis,⁴¹ while further proteolysis and amino acid sequencing of the labeled membrane proteins enable the structural analysis of the protein fragments, which provides valuable structural information on membrane proteins.

hydrophobic molecules of (trifluoromethyl)phenyldiazirine (TPD),⁴² which can be readily incorporated into native or artificial membrane environments, have been used frequently to study membrane proteins. More recently, photoactivatable amino acids have also been used to investigate protein–protein interaction in living cells.⁴³ However, the free diffusion of the small probes in the lipid domain of the membrane led to considerable nonspecific labeling.^{44–48} Efforts were thus directed to using lipid probes, which can be inserted within the membrane bilayer. Consequently, a considerable number of lipid probes with different photoactivatable chromophores have been developed over the past years. These probes are mainly derived from phospholipids, glycolipids, and sterols as all of them are important constituents of cell membranes and play critical roles in many biological processes to maintain the function of biomembranes.^{12,49} The photolabeling groups have mainly been designed to attach either at the polar head or in the hydrophobic part of lipids to probe the lipid/water interface and the hydrophobic membrane part of the biomembrane, respectively (Figure 5).⁵⁰ As shown in Figure 5A, the Type 1

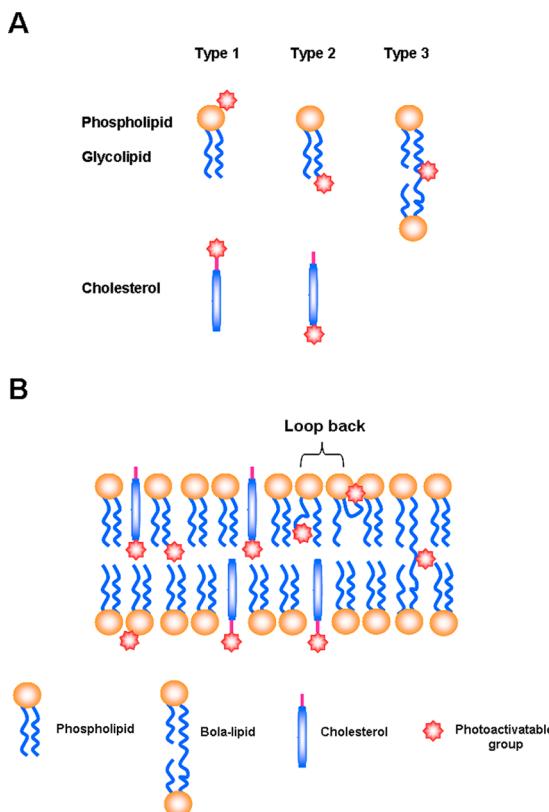


Figure 5. (A) Schematic presentation of different photoactivatable lipid probes used for investigating biomembranes. (B) Type 3 probes were developed to avoid the drawback of Type 2 phospholipid probes, which tend to loop back to probe the regions close to the polar head.

probes have the photoactive group on the polar head of the lipid for lipid/water interface labeling, whereas the Type 2 probes bear the chromophore at the lipid hydrophobic part for membrane core labeling. Noteworthy is that impaired labeling efficiency and membrane core specificity can occur with Type 2 phospholipid probes, which tend to loop back to probe the region close to the polar head due to thermal fluctuations of the fatty acyl chains above the phase-transition temperature (Figure 5B).⁵⁰ Bola-phospholipid probes (Type 3 in Figure 5A) were

therefore designed to overcome these drawbacks. These probes can span the membrane bilayer via a covalently linked fatty diacyl chain to which the photolabeling group is attached. This allows the robust insertion of the bola-lipid probes into the membrane as well as the selective and specific labeling of the hydrophobic transmembrane domain. Moreover, the incorporation of bola-lipids increases the mechanical stability and reduces permeability of biomembranes, which further potentiate the application of these probes.^{51–53}

The information provided by the photolabeling approach on the distribution patterns, structures, and functions of membrane proteins as well as lipid–protein interactions in biomembranes largely depends on the choice of photoactivatable lipid probe. In this regard, we will present in this Review a comprehensive overview of the development of photoactivatable lipid probes and their applications in photoaffinity labeling studies of biomembranes.

2. LIPID PROBES WITH DIFFERENT REACTIVE SPECIES FOR PHOTOLABELING

The concept of photoaffinity labeling was first proposed by Westheimer et al. in 1962⁵⁴ and was later implemented by Khorana and co-workers to study biomembranes.²⁹ Nowadays investigations on biomembranes require primarily lipid probes containing photoactivatable groups. There are five commonly used photolabeling chromophores, azides, diazo compounds, diazirines, diazonium salts, and diaryl ketones, which can be classified according to their photochemically generated reactive species: nitrenes, carbenes, carbocations, and radicals (Figure 6).⁵⁵ Azides, especially aryl azides, represent the dominant photolabeling reagents yielding nitrenes. Both diazo and

diazirine compounds are capable of producing carbenes as reactive intermediates. Aryldiazonium salts are often the source of the carbocations used in photolabeling, whereas the carbonyl groups present in benzophenone chromophores usually supply the radicals employed as the reactive species.

The reactivity of the photoactivatable probes relies primarily on the nature of the chemical entity. Measuring the lifetime of the photogenerated reactive species is one method of choice to evaluate the reactivity. The lifetimes of reactive intermediates greatly depend on the chemical entity and the corresponding decoration in its structure, as well as the choice of solvent, temperature, pH, etc. Although difficult to obtain the lifetimes for all individual species, the commonly used photoactive species named above, nitrenes, carbenes, and radicals have lifetimes ranging from microsecond to nanosecond,³⁷ and the most active aryl carbocations have shorter lifetimes in the picosecond range.^{56,57} Phenylnitrenes are presumed to display longer lifetimes than carbenes, allowing more translational diffusion and consequently increasing the probability of contact with target residues. The lifetimes of radicals derived from benzophenone chromophores are even longer than those of nitrenes, and these radicals display a very low reactivity toward water, preferentially abstracting the hydrogen on carbon atoms to form cross-links with target proteins.^{34,58} Photoactivatable lipid probes are mainly used for studying biological membranes, and it is believed that the hydrophobic interior of biomembranes provides far more favorable medium for photolabeling than an aqueous medium. Indeed, the relative chemical inertness of the phospholipid bilayer can increase the lifetime of the reactive species and ensure their chance of attacking membrane proteins successfully,³⁰ notwithstanding the exact impact of apolar core on the individual photoreaction. It is worth mentioning that the shorter half-life of the reactive species is also desirable to minimize nonspecific labeling. It is hence important to choose the suitable photoactivatable groups that generate reactive species with the desired lifetime for efficient labeling.

The first photoactivatable lipids, synthesized by the group of Khorana in the 1970s, contained azido (and also diazo) groups at different positions on the lipids.²⁹ Their work demonstrated that synthetic phospholipid probes, like their natural counterpart, could also form vesicles and that photolysis of these vesicles resulted in the formation of higher molecular weight products indicating the intermolecular cross-linking of fatty acid chains. Since then, a variety of photoactivatable lipid probes involving phospholipids, glycolipids, and sterols have been developed, and they have constituted useful tools to get deep insight of biomembranes. These lipid probes can be chemically classified according to the reactive species generated from the different photoactive chromophores located in the lipid scaffold. Alternatively, they can be assorted according to the location of the chromophore within the lipid structure, either at the lipid polar head or in the hydrophobic region. Here, we will report on the lipid probes according to the reactive species generated upon photoirradiation.

2.1. Nitrenes

The most common precursors used to deliver nitrenes in photolabeling studies are azides (Figure 6). This is mainly because of the small size of the azido group and the relative ease with which it is introduced into the parent molecule, along with its chemical stability in the dark yet high reactivity upon photoirradiation. Aryl azides are widely used in photolabeling,

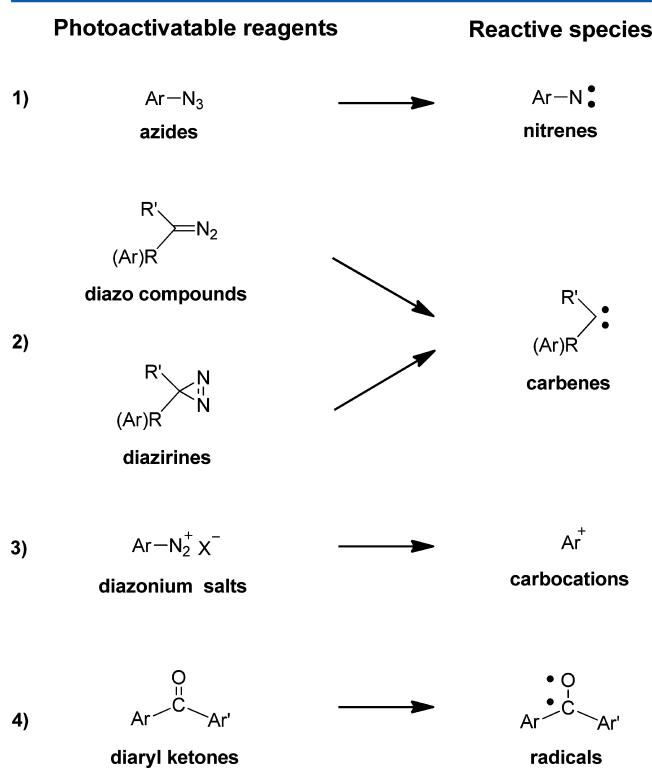


Figure 6. The commonly used photolabeling chromophores (azides, diazo compounds, diazirine, diazonium salts, and diaryl ketones) and the corresponding reactive species (nitrenes, carbenes, carbocations, and radicals) generated upon photoirradiation.

whereas the alkyl azides have limited applications due to their poor stability even though they can also generate nitrenes.⁵⁵ The first aryl azide probe **1** for photolabeling study was synthesized by Knowles and co-workers in 1969 (Figure 7).⁵⁹

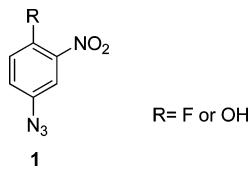


Figure 7. The first aryl azide photoactivatable reagent **1** synthesized by Knowles et al.⁵⁹

Khorana et al. then reported the incorporation of either an alkyl azido or an aryl azido group in phospholipids (compounds **2–6** in Figure 8) to unravel lipid–protein interactions, which further expanded the biological application of azide compounds in photoaffinity labeling.²⁹

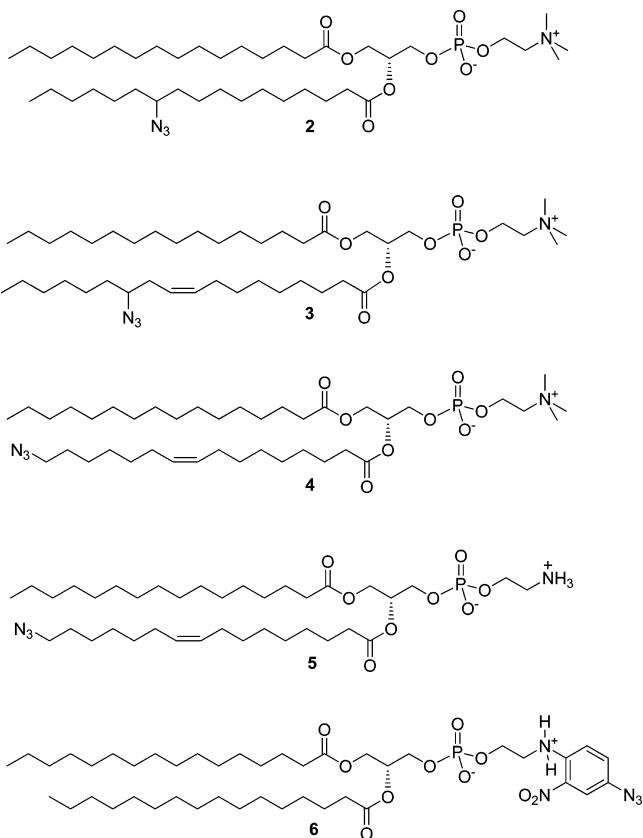


Figure 8. The first phospholipid probes **2–6** containing an azide chromophore were developed by the group of Khorana to decipher lipid–lipid interactions in biomembranes.²⁹

Following the pioneering work of Khorana, various photolabeling phospholipid probes harboring azido groups were invented. In 1980, Brunner et al. synthesized the *p*-azidophenyl group containing phosphatidylcholine (PC) probes **7** and **8** (Figure 9) to study gramicidin A.⁶⁰ Next, Schroit et al. introduced the ¹²⁵I labeled phenyl azido group to the fatty acyl chain of the PC (**9** in Figure 9) to explore their interaction with human erythrocyte membrane proteins.⁶¹ Gao et al. went on to construct the ¹²⁵I labeled photoactivatable phosphatidylethanolamine (PE) probe **10** (Figure 9) bearing the phenyl azido at the

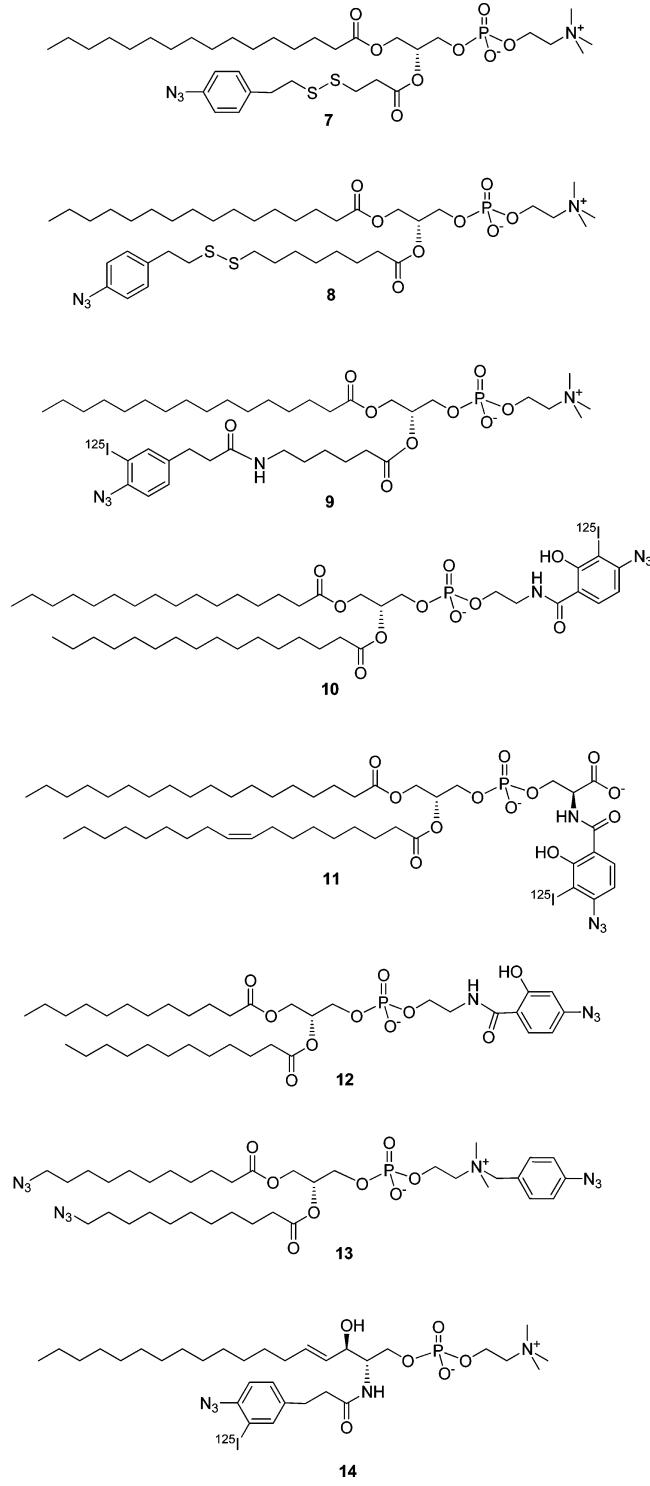


Figure 9. The photoactivatable phospholipid and sphingolipid probes **7–14** bearing an aryl azido group.^{60–65}

polar head.⁶² This probe selectively incorporated into the outer leaflet of the lipid bilayer, and the subsequent photolabeling study led to the successful identification of the subunits of ATP-synthase in thermophilic bacterium PS3.⁶² The photoactivatable phosphatidylserine (PS) probe **11** (Figure 9) then was created by Blanton et al. to investigate the *Torpedo californica* nicotinic acetylcholine receptor (AChR),⁶³ and the lipid probes **12**⁶⁴ and **13**⁶⁴ (Figure 9) were recently developed by Gubbens et al. It is worth mentioning that the azido groups

attached to the terminals of acyl chains in probe **13** are not for photolabeling purpose, but rather for facilitating analysis of labeling products via biotin conjugation through click chemistry.⁶⁴ In addition to various PE, PC, and PS probes, the sphingolipid probe **14** (Figure 9) was also synthesized to identify the proteins involved in sphingolipid sorting or metabolism in mammalian cells.⁶⁵ This photoactivatable sphingolipid analogue was metabolically active and could be transported into and metabolized by the cells, highlighting its potential for cell biological studies.

Unsubstituted phenylazides have their maximal absorption around 260 nm, implying that the most effective photoactivation will be achieved at 260 nm. However, irradiation at this wavelength may cause considerable damage to biomacromolecules such as proteins and nucleic acids because they also have absorption maxima around 260–280 nm due to the presence of aromatic amino acid residues and aromatic nucleobases, respectively. Investigations on the effects of introducing different substituents on aryl azides revealed that a nitro substitution can shift the absorption wavelength beyond 300 nm.⁶⁶ This would be expected to have a beneficial effect because photoirradiation at 300 nm or above is significantly less destructive to biomacromolecules. Molinari et al. thus attached a nitro-substituted arylazido group to different positions on the fatty acyl chain to obtain probes **15** and **16** (Figure 10) to study

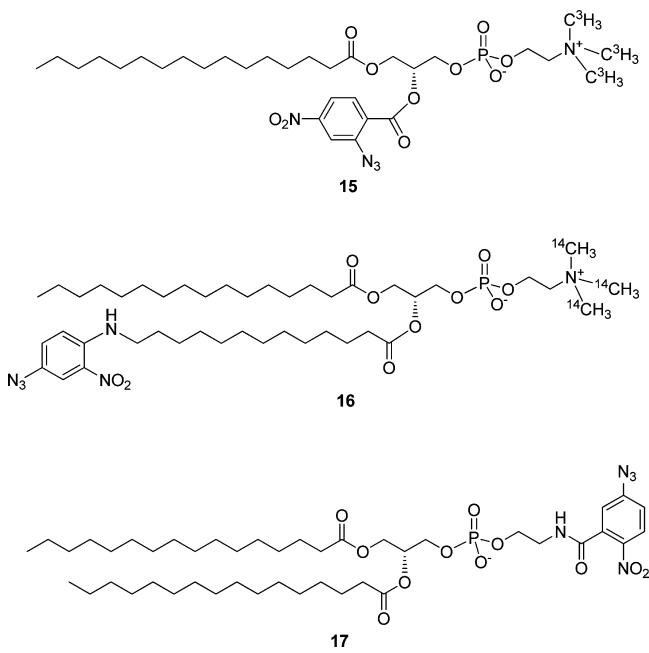


Figure 10. The photoactivatable phospholipid probes **15–17** bearing a nitrophenyl azide.^{67,68}

the binding mode of toxin VacA toward the host cell, as well as the penetration of this toxin in the lipid bilayer.⁶⁷ The probes **6** (Figure 8)²⁹ as well as **17** (Figure 10) developed by Peng⁶⁸ also represent examples of such a beneficial effect.

The photochemistry of aryl azides has been extensively investigated for the purpose of photolabeling (Figure 11).^{32,69,70} Upon photoirradiation, the azide is activated to form a singlet nitrene (S_1) accompanied by the release of nitrogen. The singlet nitrene (S_1) preferentially undergoes bond insertion into carbon–hydrogen or heteroatom–hydrogen bonds (pathway A), but also ring expansion and

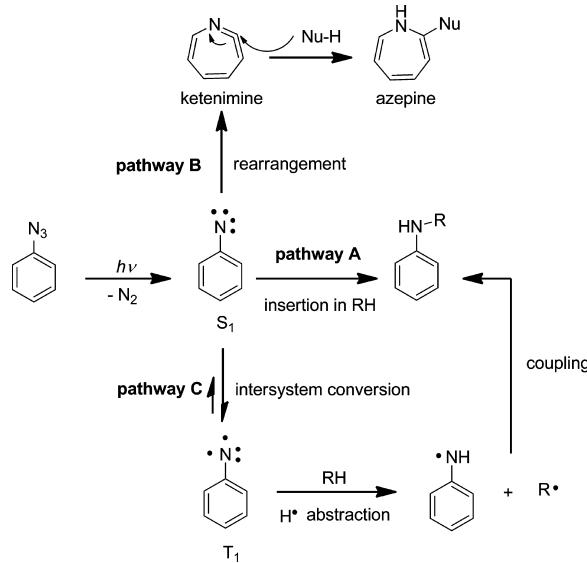


Figure 11. The photochemical reaction pathways of phenyl azide.⁶⁹

subsequent rearrangement to ketenimine (pathway B), or formation of a triplet intermediate (T_1) via the intersystem conversion (ISC) (pathway C) (Figure 11). Only pathway A will yield an efficient photolabeling because, in pathway B, the ketenimine preferentially reacts with nucleophiles to generate azepine, and, in pathway C, the triplet nitrene requires the abstraction of a hydrogen radical for coupling to produce the insertion product (Figure 11). Consequently, a successful photolabeling depends on achieving a reactive yet stable singlet nitrene (S_1), which possesses a reasonably long lifetime to favor the insertion reaction (pathway A) while avoiding ring expansion (pathway B) and/or the formation of a triplet nitrene (T_1) (pathway C).

One of the drawbacks of using aryl azides as photolabeling reagents is that the generated singlet nitrene not only undergoes an insertion reaction for the labeling but also easily rearranges to the ketenimine, which only reacts with nucleophiles (hydroxyl, amino, hydrosulfide groups, etc.), thus decreasing the photolabeling efficiency.⁷¹ The advent of fluorinated aryl azides is expected to overcome this limitation because the electron-withdrawing fluorine substituents can favor the reactivity of aryl nitrene for bond insertion,⁷² while at the same time impair ring expansion when compared to their nonfluorinated counterparts.⁷³ Keana et al. confirmed that the carbon–hydrogen or nitrogen–hydrogen insertion were the main photoproducts of the fluorinated phenyl azides upon irradiation and that ring expansion was significantly retarded (Figure 12).⁷⁴ Likewise, Platz and co-workers reported that the C–H insertion of fluorinated aryl nitrenes was almost quantitative.⁷⁵ Today, fluorinated aryl azides represent the most promising aryl azide probes available for photoaffinity labeling studies.

Platz and co-workers went on to discover that the number and position of fluorine substitutions on the phenyl ring had a profound impact on the photochemical reaction.⁷⁶ In their study, two fluorine substituents at *ortho* and *ortho'* positions relative to the azide group played crucial roles in retarding the ring expansion and stabilizing the singlet nitrene. In addition, the photolysis of fluorinated aryl azides preferentially produced singlet nitrenes in a hydrophobic environment, thus implying the potential use of this photolabeling reagent to label the

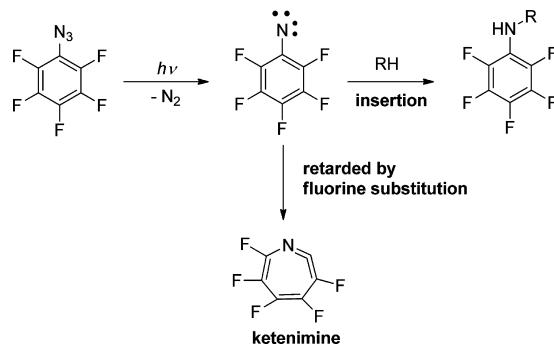


Figure 12. The photochemical reaction of fluorinated phenyl azide.⁷⁴

hydrophobic region in biomembranes. It is also worth noting that the presence of fluorine in the photolabeling reagents permits monitoring the labeling process using ¹⁹F NMR,^{77–79} thus creating new opportunities to investigate the structural and dynamic properties of biomembranes.⁸⁰

To capitalize on all of these advantageous features of fluorinated aryl azides, Peng et al. developed two phospholipid probes, **18** and **19** (Figure 13), possessing the tetrafluorophenylazido chromophore either at the polar head via amide linkage or in the fatty acyl chain via ester linkage, respectively.^{81,82} More recently, Xia et al. introduced the tetrafluorophenylazido group to the phospholipids via more stable and reliable amine and ether functionalities (**20** and **21** in Figure 13) as compared to the alternative amide and ester moieties.⁸³ Here, the amine function in **20** was expected to retain the protonation ability lost by the amide, and the ether linkage in **21** to confer greater stability and better mimic the alkyl chain as compared to the ester group in **16**. Interestingly, **20** exhibited unusual ¹⁹F NMR signals that depended on the protonation state of the amino group at the polar head.⁸³ This can be exploited to examine various pH-dependent events in biomembranes.^{84,85} Moreover, a fluorinated phenyl azide was also used by Huang et al. in the synthesis of the photoactivatable analogues of hexadecylphosphocholine (miltefosine) with a tetrafluorophenylazido chromophore incorporated either at the polar head (**22** in Figure 13) or in the long alkyl chain (**23** in Figure 13).⁸⁶ It is worth mentioning that miltefosine is an alkylphosphocholine showing remarkable antineoplastic, antitumor, and antileishmanial activities.⁸⁷

Aside from the above-mentioned probes, the bola-lipid probes (Figure 5) are particularly appealing for studying biomembranes due to their unique structure and specific labeling of the transmembrane domain. Xia et al. therefore synthesized the photoactivatable bola-phospholipid probe **24** (Figure 14) harboring the fluorinated aryl azido group at the central point of the two covalently bonded chains via a thioacetal linkage.^{88,89}

In addition to phospholipids containing azido groups, glycolipids bearing this photoactivatable moiety have also been reported. Some glycolipids behave as anchors to attach cell surface proteins to the cell membrane; an example is glycosyl phosphatidylinositols (GPIs), which are synthesized by the sequential addition of sugars and other components to phosphatidylinositols (PIs) in the endoplasmic reticulum (ER).^{90,91} In an attempt to explore the molecular and structural basis of GPI-anchored proteins, Mayer et al. synthesized a series of photoactivatable probes **27–29** based on the glycosyl phosphatidylinositol **25** (Figure 15),⁹² which plays a prominent role in GPI anchor biosynthesis. All of the probes **27–29** have

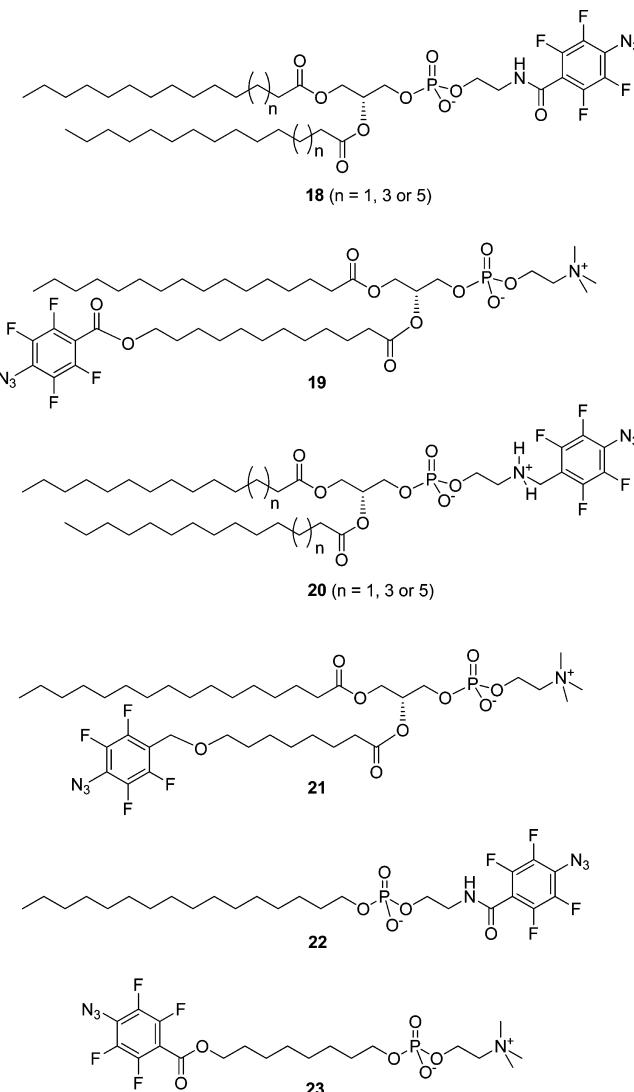


Figure 13. The lipid probes **18–23** containing a tetrafluorinated phenyl azido group.^{81–83,86}

the aryl azido group attached at the carbohydrate part. Probe **29** is radiolabeled with ¹²⁵I to provide sensitive detection and easy analysis of the labeled products in trace amounts.

Although the introduction of the fluorine atom did greatly improve the photolabeling efficiency of the aryl azides, these photolabeling reagents still suffer from some drawbacks: (1) The maximum absorption wavelengths of aryl azides are usually around 260 nm, and irradiation at this wavelength may lead to considerable damage to biomacromolecules, whereas above this wavelength it will yield neither sufficient nor desirable labeling. (2) Nitrenes generated from the azides are normally less reactive than carbenes and carbocations for labeling of hydrophobic environment.⁹³ (3) The insertion reaction of nitrenes yields carbon–nitrogen or labile heteroatom–nitrogen bonds, which may not be robust enough to undergo the postlabeling treatment, hence complicating the analysis of photolabeling products. Fortunately, other photoactivatable chromophores, such as diazirines, diazo compounds, and diaryl ketones, can compensate for some of these limitations, and hence enrich and diversify the choice of available photolabeling lipid probes for investigating biomembranes.

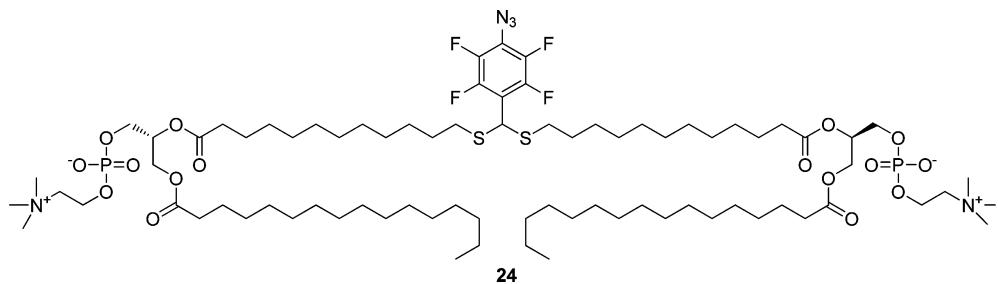


Figure 14. The bola-phospholipid probe **24** containing a fluorinated phenyl azide.^{88,89}

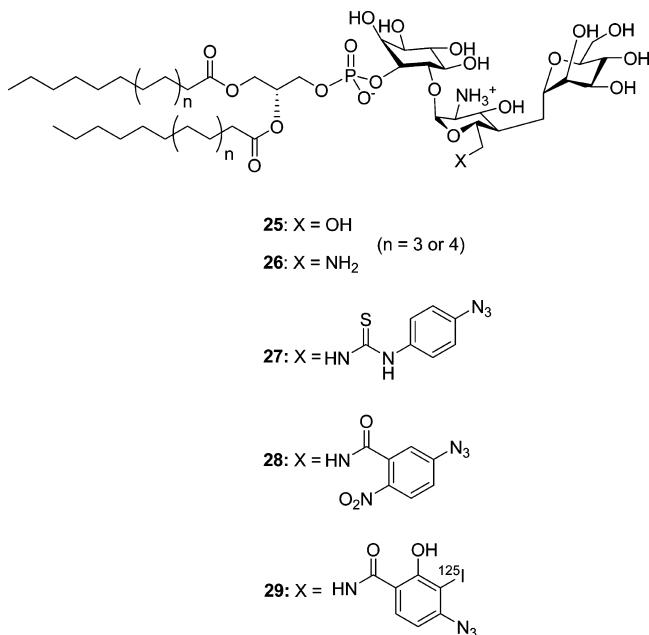


Figure 15. Glycosyl phosphatidylinositol (GPI) **25**, its amino derivative **26**, and the corresponding photolabeling probes **27–29**.⁹²

2.2. Carbenes

Carbenes as reactive intermediates have established their uniquely important place in photolabeling. More reactive than nitrenes, carbenes not only insert into carbon–hydrogen or oxygen–hydrogen bonds to form more stable carbon–carbon or carbon–oxygen bonds, but also react with diverse nucleophiles. Diazo and diazirine compounds represent the major carbene precursors (Figure 6), and the implementation of both in photolabeling lipid probes will be illustrated below.

2.2.1. Diazo Compounds. Similar to azido function, the diazo group is small in size and easy to introduce. As such, the first example of photoaffinity labeling was achieved with a diazoacetate and performed by Westheimer and co-workers.⁵⁴ Later, diazo ketones were developed as photolabeling reagents thanks to their favorable photochemical properties. Upon irradiation, diazo ketones **30** (Figure 16) are decomposed to produce the highly reactive carbene accompanied by the release of molecular nitrogen. This carbene product can undergo a photolabeling process by bond insertion. However, it may also undergo the Wolff rearrangement to yield a ketene intermediate, which reacts with nucleophiles (Figure 16) and thus negatively impacts on accurate photolabeling.⁵⁵ Nevertheless, the introduction of electron-withdrawing groups was shown to reduce such Wolff rearrangement. This has been confirmed by Chowdhry et al. with data obtained from the

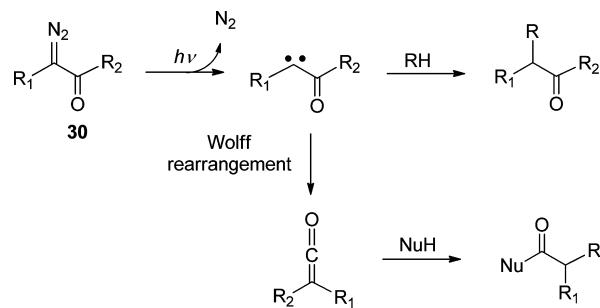


Figure 16. The photochemical reaction of diazo ketones for photoaffinity labeling.⁵⁵

photolysis of ethyl diazotrifluoropropane **31** in methanol, which yielded much less rearrangement products, thus allowing for an efficient insertion into the O–H bond (Figure 17).⁹⁴

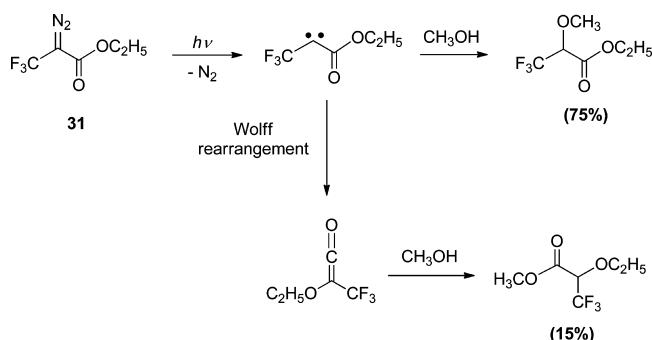


Figure 17. The photochemical reaction of the ethyl diazotrifluoropropane **31** yielded considerably decreased levels of the Wolff rearrangement product.⁹⁴

In 1983, Takagaki et al. reported the phospholipid probe **32** bearing a trifluoromethyl diazo group (Figure 18) to label cytochrome *b5*.⁹⁵ As diazocyclopentadiene displayed improved stability over typical alkyl diazo compounds, Uvarov et al. imported the diazocyclopentadiene into the phosphocholine and obtained probes **33** and **34** (Figure 18) with the aim of scrutinizing the membrane-bound sites of cytochrome P-450 2B4 at different depths.⁹⁶ Later, Alcaraz et al. developed the phospholipid probe **35** endowed with the diazocyclohexadienylnonyl moiety (Figure 18), which displayed high stability and a maximal UV absorption around 360 nm, highlighting its promising potential for use in photolabeling studies.^{31,97}

2.2.2. Diazirines. Like diazo compounds, diazirines can generate highly reactive carbenes when exposed to light or thermolysis (Figure 6). As shown in Figure 19, the diazirine is activated by photoirradiation to form the reactive carbene alongside the corresponding diazo product, which undergoes

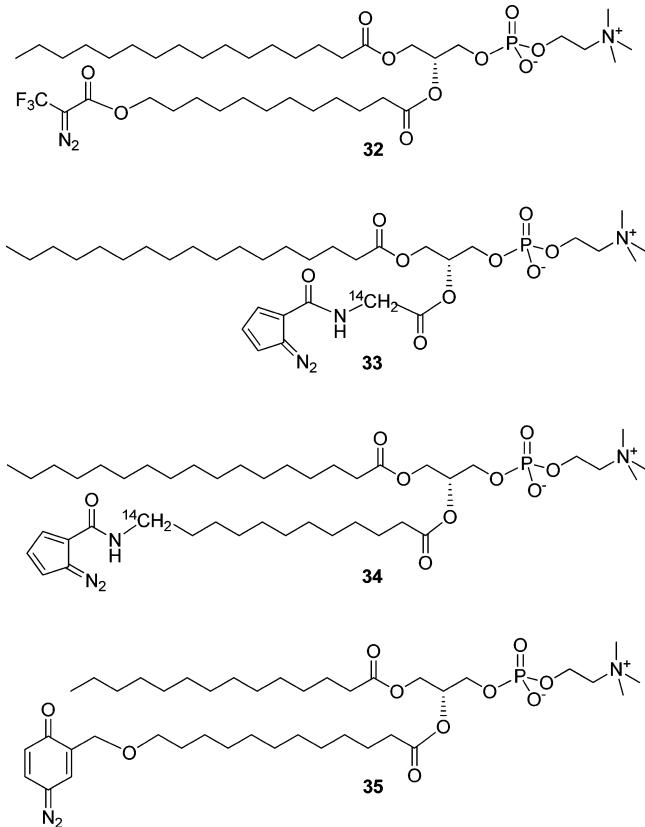


Figure 18. The phospholipid probes 32–35 bearing diazo groups.^{95–97}

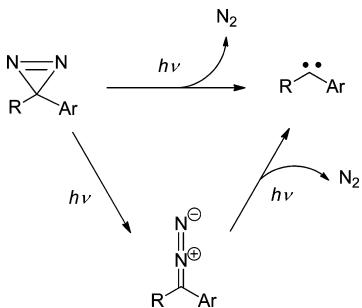


Figure 19. The photochemical reaction of diazirines to generate reactive carbene species for photoaffinity labeling.⁵⁵

further photolysis to produce more carbene.^{55,98} Moreover, the relatively thermal and chemical stability of the aryl diazirines contributes to their wide range of applications in photolabeling studies of biological and synthetic macromolecular systems.^{98,99}

Knowles et al. initially used aryl diazirines as potential reagents for photolabeling biological receptors¹⁰⁰ and then biomembranes.¹⁰¹ Soon after, Brunner et al. developed the most potent diazirines for photolabeling, the aryl-trifluoromethyl diazirines (Figure 20).⁴² As compared to other diazirines, the aryl-trifluoromethyl diazirines offer much better chemical and thermal stability thanks to their adjacent aryl and trifluoromethyl groups.⁹⁸ The corresponding carbenes do not undergo rearrangement such as Wolff rearrangement shown in Figure 16, hence avoiding the unintended labeling of the rearrangement products (Figure 20).⁴² Moreover, as the maximal absorption of aryl-trifluoromethyl diazirines is observed around 360 nm, activation at this wavelength considerably

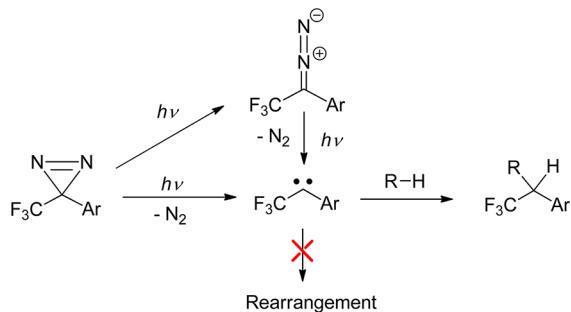


Figure 20. The photochemical reaction of aryl-trifluoromethyl diazirines for photolabeling.⁵⁵

avoids photodamage to biomacromolecules. Collectively, aryl-trifluoromethyl diazirines are currently regarded as the most efficient photolabeling reagents developed so far, and their use in studying the biomembranes will be exemplified in section 5.

Because carbene-based photoaffinity labeling is more efficient in hydrophobic environments as compared to in aqueous medium,⁹⁸ various hydrophobic yet small molecular probes containing diazirine chromophores have been developed for the labeling of biomembrane hydrophobic regions.^{44,98,101} However, the random distribution of these probes in the lipid bilayer often leads to nonspecific labeling.³⁰ Incorporation of these molecular probes into the lipid skeleton is expected to overcome this pitfall.^{30,98} Accordingly, Ross et al. synthesized the lipid probes 36–38 bearing diazirine either in the fatty acyl chain or at the polar head (Figure 21).¹⁰² These probes have been successfully applied to study the transmembrane domain of glycophorin A.¹⁰² Phospholipid probe 39 bearing diazirine in the fatty acyl chain (Figure 21) was generated biosynthetically using diazirine-bearing stearic acid to label synaptic-like microvesicles (SLMVs).¹⁰³ Furthermore, lipid probes 40–43 containing aryl-trifluoromethyl diazirine were also created (Figure 21).^{60,64,104,105} It is to note that probe 41 has a S–S bridge in the fatty acyl chain, which can be cleaved after the photolabeling study to generate a free thiol group with the aim of facilitating the subsequent analysis of the labeled peptides.⁶⁰ Additionally, the diazirine-bearing derivatives of ET-18-OCH₃, 44 and 45 (Figure 22),¹⁰⁶ were synthesized to study the mechanism of action of ET-18-OCH₃, a phosphocholine analogue with anticancer activity (Figure 22).^{107,108} Probe 45 was found to display antiproliferation activity similar to that of ET-18-OCH₃, suggesting that this photoactivatable probe is able to mimic the function of ET-18-OCH₃ and may thus be appropriate for photolabeling studies to identify proteins interacting with ET-18-OCH₃. In 1993, Delfino et al. first constructed the membrane-spanning bola-lipid probe 46 (Figure 23), which has a centrally defined attachment point for the (trifluoromethyl)phenyldiazirine as the photolabeling group.⁵⁰ This probe is chemically stable and can be processed efficiently upon photolysis with light of wavelengths above 300 nm. It is worth mentioning that the use of thioacetal linkage to introduce the chromophore in the middle of the diacid chain for the bola-lipids is of interest for its chemical stability and synthetic ease under mild conditions. Additionally, the relatively low polar character of the thioacetal bridge in probes 24 and 46 may effectively mimic the aliphatic chain because the presence of polar heteroatoms within the photoactivatable fatty acyl chain is able to increase the transfer of the phospholipid between membranes.¹⁰³

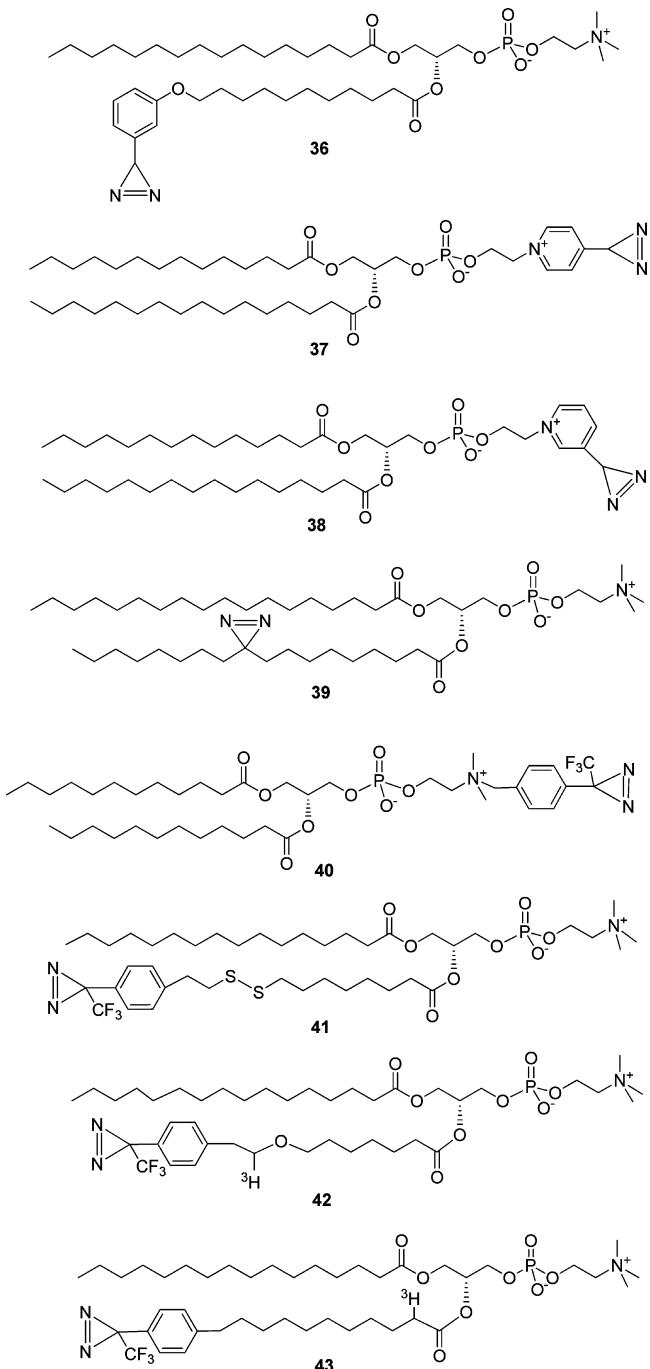


Figure 21. Phospholipid probes 36–43 containing a diazirine moiety.^{60,64,102–105}

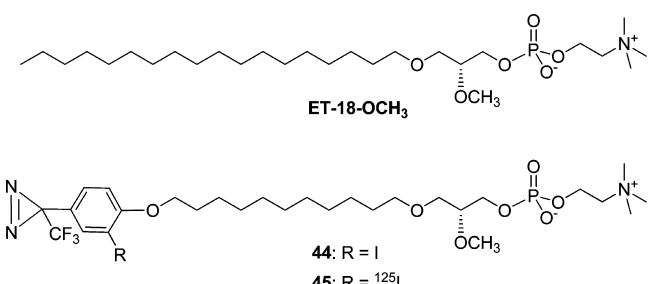


Figure 22. ET-18-OCH₃ and its photoactivatable analogues 44 and 45.¹⁰⁶

While diazirines appear to be the most promising chromophores for photoaffinity labeling, introducing them into different bioactive molecules in a simple and convenient manner remains challenging and constitutes the major impediment to their practical use.^{98,99} Much inspirational and enthusiastic input from synthetic chemists in this regard is now essential to promote the application of diazirine-based photoprobes.

2.3. Carbocations

Besides nitrenes and carbenes, carbocations also belong to the group of highly reactive photochemical species.⁵⁶ By virtue of their higher activity, carbocations have stronger capacity to form cross-linkages than nitrenes or carbenes. Probes based on carbocations are thus expected to yield more robust photolabeling. Aryldiazonium salts are usually used to generate carbocations in photolabeling reactions.^{32,55} Figure 24 displays the photochemical reaction of aryldiazonium salts: upon photoirradiation, the aryldiazonium salts are decomposed to form highly reactive aryl cations with a concomitant release of molecular nitrogen. The generated aryl carbocations subsequently undergo reactions with various X–H bonds (X = C, O, N, S). Many aryl diazonium salts are not very stable under normal light condition. It is to note that electron-withdrawing groups on the aryl chromophores usually make the diazonium salts less stable, while electron-donating substituents confer the stability that favors their application in photolabeling.^{32,109}

Examples of conjugating the aryldiazonium chromophore to the phospholipid skeleton are very limited: this is mainly due to the poor stability of aryldiazonium salts. Compound 47 in Figure 25 is a phospholipid carrying the aryldiazonium group at the polar head of the phospholipid.^{31,97} Probe 47 has the advantage of being activated via tryptophan-mediated energy transfer as its absorption maximum is close to the fluorescent emission wavelength of tryptophan. This allows specific labeling of the regions within close vicinity of the tryptophan residues in proteins.³¹ This probe was developed by Goeldner and co-workers, a research group possessing considerable experience in the use of aryldiazonium salts for protein labeling,³² and especially in employing aryldiazonium salts to mimic cholinergic ligands and probe the binding sites of cholinergic proteins.

2.4. Radicals

Free radicals represent another important class of highly reactive intermediates in photoaffinity labeling. Benzophenone derivatives are the most widely used reagents to produce the reactive free radicals useful in photolabeling.⁵⁵ The photochemical reaction of benzophenone is depicted in Figure 26. Upon photoirradiation, benzophenone is activated to form a triplet biradical, which then abstracts the hydrogen atom from a nearby molecule to transform it into a free radical and at the same time form the H-containing radical species derived from benzophenone. Subsequent recombination of the two radicals leads to the photoadduct.

By their preferential reaction with unreactive C–H bonds, even in the presence of solvent water,¹¹⁰ benzophenone probes are valuable for producing efficient covalent modifications of macromolecules. Galardy et al. first applied benzophenone to synthesize the pentagastrin probe and with it labeled the binding sites of the hormone pentagastrin on bovine serum albumin.¹¹¹ Since then, benzophenone chromophores have been used to functionalize diverse bioactive molecules with the

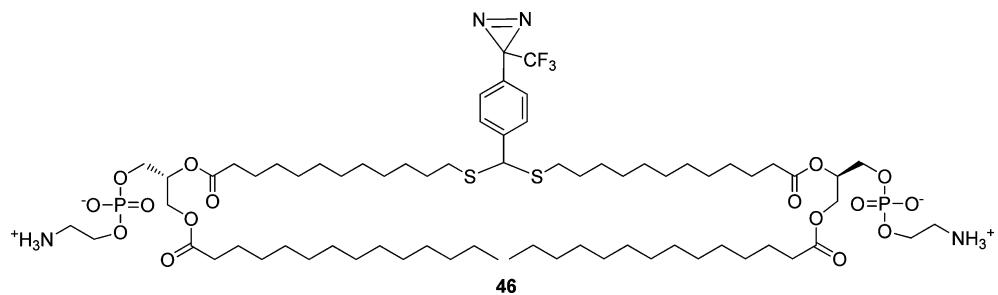


Figure 23. Bola-lipid probe **46** containing an aryl-trifluoromethyl diazirine moiety.⁵⁰

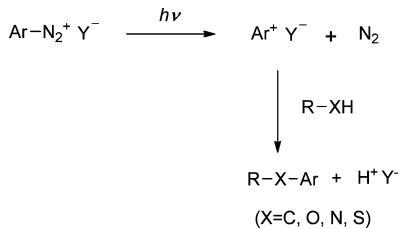


Figure 24. The photochemical reaction of aryl diazonium salts for photoaffinity labeling.

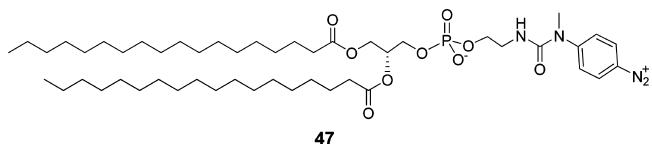


Figure 25. The phospholipid probe **47** containing an aryl diazonium moiety.⁹⁷

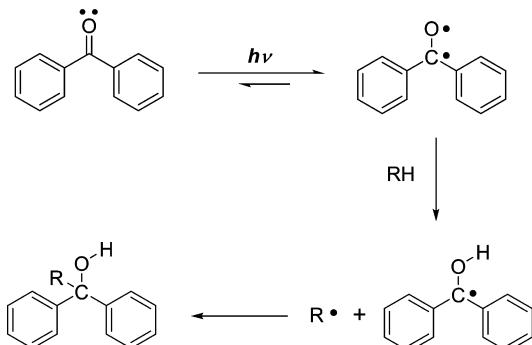


Figure 26. The photochemical reaction of benzophenone for photolabeling.⁵⁵

intention of probing the interaction of these parent molecules with their biological receptors.^{110,112}

Benzophenone derivatives offer several advantages as photolabeling reagents relating to their (1) elevated chemical stability and relatively easier synthesis as compared to azides, diazirines, and diazo compounds; (2) reduced sensitivity to ambient light yet capacity to be activated at wavelengths above 350 nm to avoid photodamage to biomacromolecules; and (3) preferential reactivity with unreactive C–H bonds and lower reactivity in solvent water.¹¹⁰ Since the 1980s, various phospholipid probes endowed with benzophenone chromophores have been prepared for photoaffinity labeling of biomembranes. Probe **48** (Figure 27) reported by Montecucco et al. was used to study Na⁺, K⁺, and Ca²⁺-activated ATPase,¹¹³ and all of the proteins labeled by this probe had a known integral nature, highlighting

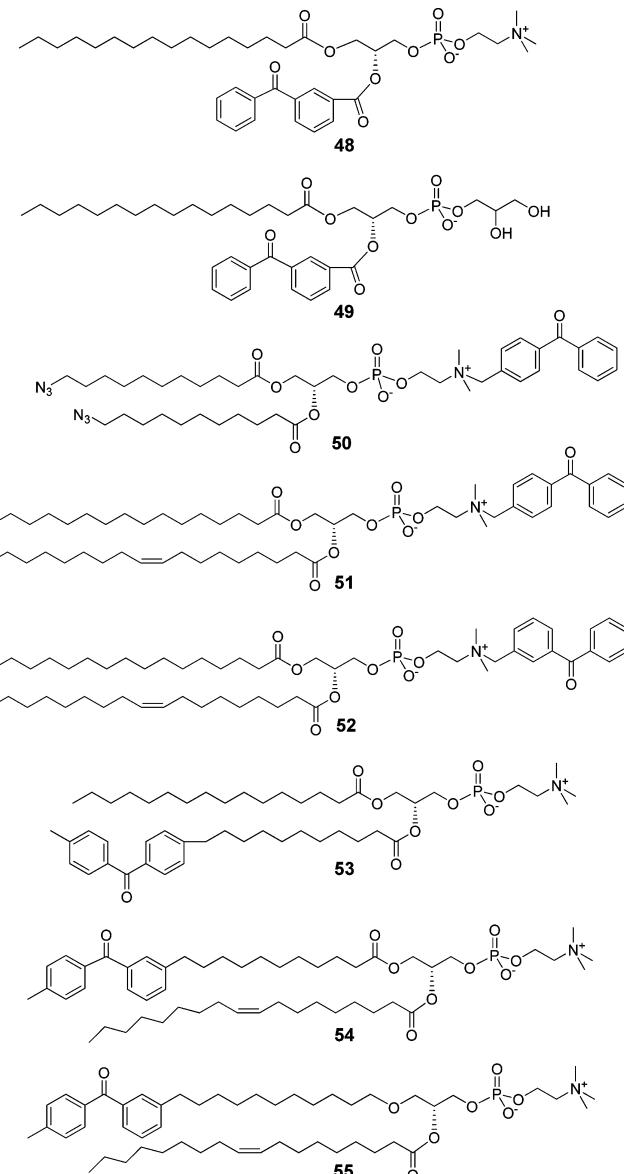


Figure 27. The phospholipid probes **48–55** containing benzophenone chromophores.^{64,113–115}

its propensity to cross-link with intrinsic membrane proteins. In the same year, Page et al. developed the probe **49** (Figure 27) to label the pore-forming proteins on the outer membrane of *Escherichia coli*.¹¹⁴ Probe **50** (Figure 27) was constructed to study lipid–protein interactions by carrying benzophenone entity at the polar head of phosphocholine and, at the same time, two azido groups at the terminals of the fatty acid

chains.⁶⁴ These terminal azido groups can be used for the covalent attachment of an alkyne-conjugated tag via click chemistry for visualization or affinity purification, and thus facilitate postlabeling analysis.⁶⁴ In 2004, Wang et al. synthesized a series of benzophenone-containing PC analogues **51–55** (Figure 27),¹¹⁵ which harbor the photolabeling group either at the lipid polar head via a quaternary ammonium functionality or in the fatty acyl chain via an alkyl linkage. The benzophenone-based probes **56–58** (Figure 28) prepared by

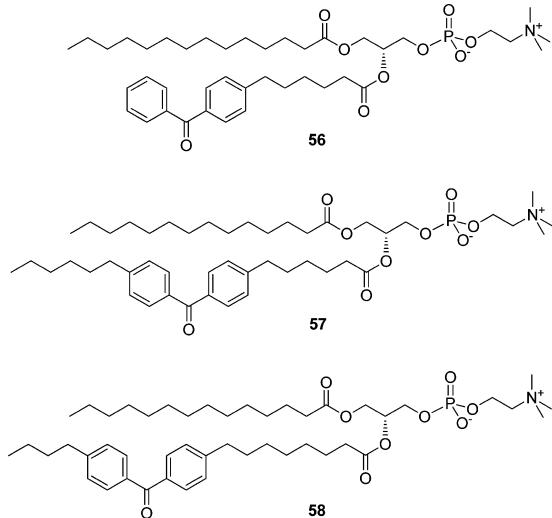


Figure 28. The benzophenone-based phospholipids **56–58** developed by Lala et al.¹¹⁶

Lala et al. have the chromophore group placed elegantly at different locations on the fatty acyl chain, thus positioning them at various depths within the lipid bilayer and envisaging their region-specific photochemical cross-linking.¹¹⁶ Besides the bola-lipids bearing aryl azido and diazirine groups we mentioned above, bola-lipid probes **59** and **60** (Figure 29) containing benzophenone were constructed by Diyizou et al.¹¹⁷ This probe could be incorporated perpendicularly within the bilayer and efficiently photolabeled neighboring lipids or/and proteins located near the middle of the membrane core with extremely high regioselectivity. Very recently, Bandyopadhyay et al. designed and synthesized a set of phosphatidylserine lipid mimics (**61–65**) with benzophenone as photoactivatable moiety (Figure 30) with view to searching for new phosphatidylserine (PS) receptors.¹¹⁸ Among these probes, an alkyne moiety in probes **61–63** was used to facilitate postlabeling readout through conjugation via click reaction.

Besides the benzophenone-based phospholipids described above, the paradigms of other lipid forms such as diacylglycerol (DAG), phosphatidic acid (PA), phosphatidylinositol polyphosphate lipids (PIP_n's), and cholesterol endowed with

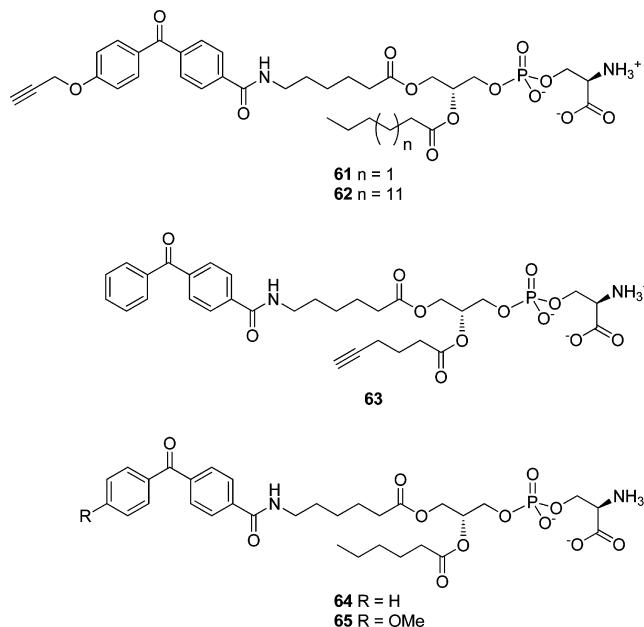


Figure 30. The benzophenone-based phosphatidylserine analogues **61–65** developed by Bandyopadhyay et al.¹¹⁸

benzophenone also have been reported. DAG, PA, and PIP_n's are signaling lipids.¹¹⁹ While they are present at low percentages in cellular membranes, they constitute crucial mediators in a wide range of biological processes. The DAG probe **66** (Figure 31) was established by Smith et al. aiming at understanding the binding interactions between the DAG and DAG-binding receptor, protein kinase C.¹²⁰ Later, the same research group developed PA probe **67** (Figure 31) containing a benzophenone as the photoaffinity moiety and an azide as a secondary handle for postlabeling purification purposes.¹²¹ In 1997, Tall et al. used PIP analogues **68,69** (Figure 31) to probe the phosphoinositide-specific phospholipase C (PLC) isoforms for evidence of multiple high affinity binding sites.¹²² In 2009, Gong et al. reported the synthesis of modular PIP_n's headgroup scaffolds that could be conveniently derivatized to a range of functionalized analogues. One of these was a bifunctional probe **70** (Figure 31) containing a benzophenone group for photo-cross-linking with cognate receptors as well as an azido group for click chemistry conjugation to speed up the postlabeling analysis.¹²³ This probe was used to purify, identify, and characterize PIP_n-binding receptors.¹²³ More recently, Rowland et al. also developed the bifunctional probes **71–73** (Figure 31) involving the headgroup of PI(3,4,5)P₃ that are effective for identifying and characterizing the PI(3,4,5)P₃ binding proteins from cell extracts.¹²⁴ These probes harbor a benzophenone group for covalent labeling of target proteins as well as the secondary tag, which can be a fluorescent dye for spectroscopic detection or an alkyne that is selectively derivatized via click

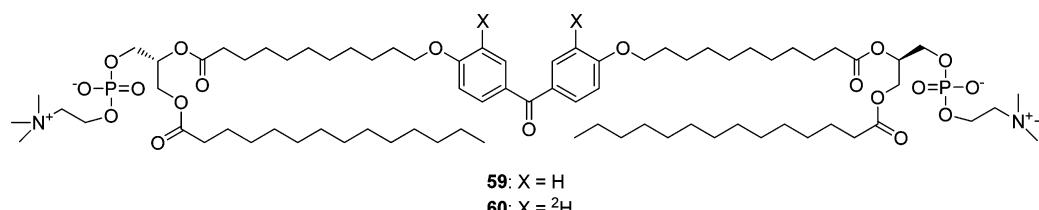


Figure 29. The benzophenone-based bola-phospholipids **59** and **60** developed by Diyizou et al.¹¹⁷

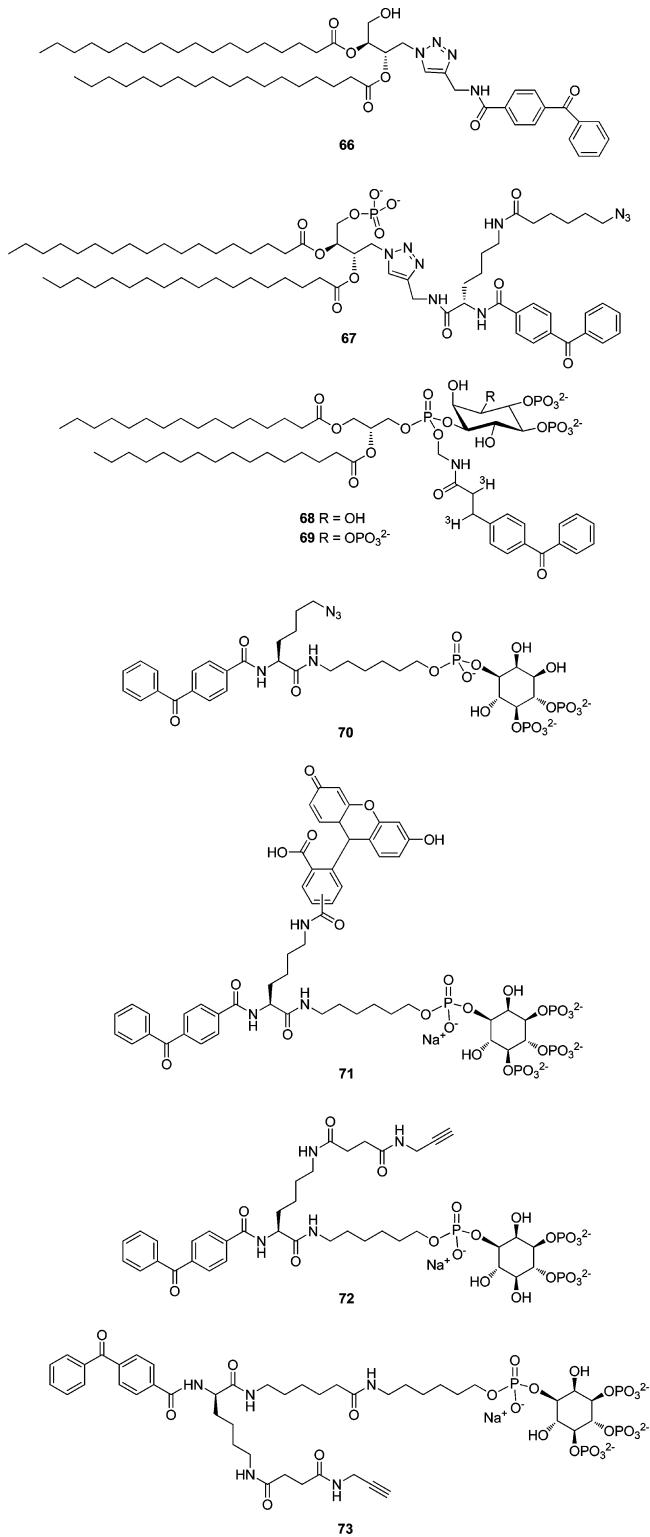


Figure 31. Photoactivatable diacylglycerol, phosphatidic acid, and phosphatidylinositol polyphosphate lipids bearing benzophenone chromophore.^{120–124}

chemistry after photolabeling to import rhodamine for in-gel detection or biotin for affinity purification.

The benzophenone-containing sterol probes emerged from around the year 2000. Fielding et al. synthesized probe 74 (Figure 32), also referred to as ^3H -FCBP, a benzophenone-modified cholesterol analogue with incorporated ^3H -radio-

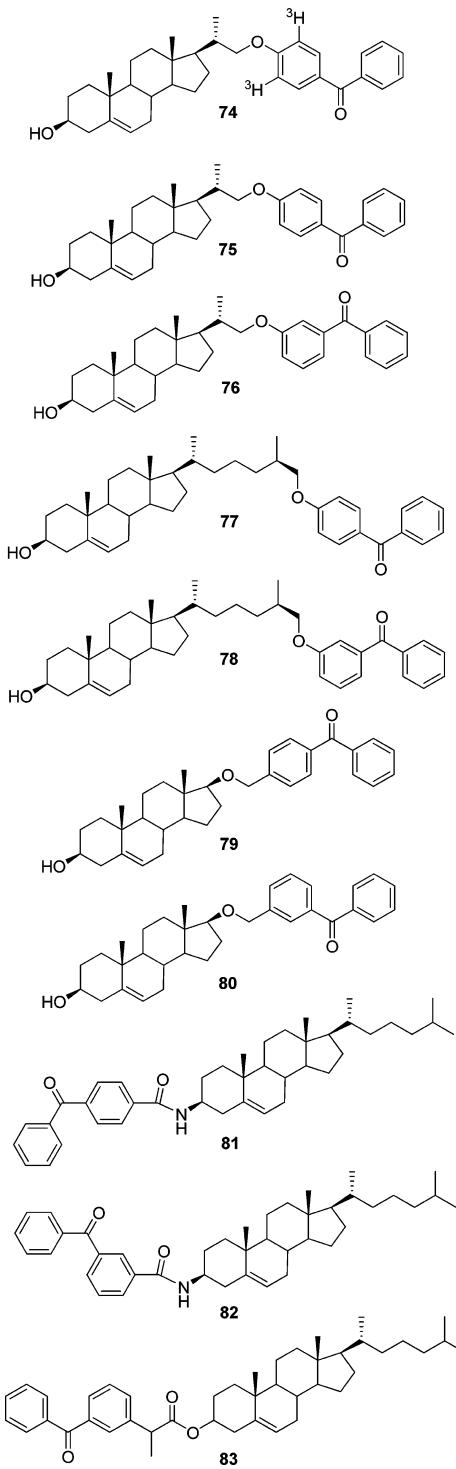


Figure 32. Photoactivatable sterol probes 74–83 bearing benzophenone chromophore.^{125–127}

activity.¹²⁵ This probe was used to investigate how the distribution of free cholesterol was affected by lipid efflux.¹²⁵ Thereafter, a multitude of cholesterol analogues carrying benzophenone chromophores were created (75–83 in Figure 32).^{126,127} Despite being loaded with bulky benzophenone chromophores, these cholesterol probes are able to incorporate efficiently into the biological membranes, preserve the ability of free cholesterol to bind proteins, and retain other major

Table 1. General Features of Photoactivatable Compounds

Compound	Photoactivatable group	Reactive species	General features
Ar—N ₃ Azides	—N ₃	Ar—N [•] Nitrenes	- Small in size; - Easy for synthesis; - Chemical stability in dark; - Maximal absorption less than 300 nm; - Form C-N or labile X-N bonds.
(Ar)R—N ₂ Diazo compounds		(Ar)R—C≡N [•] Carbenes	- Small in size; - Easy to synthesize; - Maximal absorption around 360 nm; - More active than nitrene; - Form more stable C-C or C-O bonds; - React with diverse nucleophiles; - More efficient in hydrophobic environments than in aqueous medium.
(Ar)R—N ₂ Diazirines			- Diazirines share similar properties as diazo compounds; - Aryl diazirines display improved thermal and chemical stability.
Ar—N ₂ ⁺ X [−] Diazonium salts	—N ₂ ⁺ X [−]	Ar ⁺ Carbocations	- Highly reactive and form stronger cross-linkage than nitrene and carbene; - Usually less stable; - Electron-donating substituents confer stability and absorption greater than 300 nm.
Ar—C(=O)—Ar' Diaryl ketones		Ar—C(=O) [•] —Ar' Radicals	- Increased chemical stability; - Relatively easier synthesis; - Reduced sensitivity to ambient light; - Activated at above 350 nm; - Preferential reactivity with C-H bonds; - Lower reactivity in water or with bulky nucleophiles.

properties of sterol within the cell, hence raising their appeal for use in studying the multiple intracellular steps in sterol efflux.

We have now summarized the photoactivatable lipid probes sorted by the reactive species generated upon photoirradiation of the photolabeling group. Nitrenes, carbenes, carbocations, and free radicals are the most frequently reported reactive intermediates in photoaffinity labeling. The chromophores able to produce these intermediates can be imported to the lipid structure to create various photoactivatable probes. Because different photolabeling groups endow the lipid probes with distinct photochemical properties, such as activation wavelengths, preferences toward cross-linking various chemical bonds in hydrophobic and hydrophilic environment, etc., it is thus crucial to choose the appropriate lipid probe for a specific labeling experiment based on efficacy and selectivity of the lipid probe as well as photolabeling purpose. To facilitate the reading, we have summarized the general features of different photoactive groups in Table 1 and listed all of the lipid probes cited in this Review in Table 2.

3. SYNTHESIS OF PHOTOACTIVATABLE LIPID PROBES

Regarding the synthesis of phospholipid and glycolipid probes, the principal strategy is the same whatever the kind of photolabeling group to be introduced; it involves conjugating the natural phospholipids or lysophospholipids (lyso-PC) via various chemical reactions. Because of the labile and amphiphilic features of phospholipids, conjugation reactions under mild conditions and with easy manipulation are required.

As all of the photoactivatable phospholipids or glycolipids have the photolabeling group either at the polar head or in the fatty acyl chain, two general strategies have been developed accordingly (Figure 33): (A) attaching the photolabeling group to the polar head of the natural phospholipids or sphingolipids (Figure 33A), and (B) incorporating the photolabeling group to the *sn*-2 position of glycerolipids or sphingolipids (Figure 33B).

Differing from phospholipids or sphingolipids, cholesterol is a lipid involving a polar hydroxyl group, four fused hydrophobic rings, and a hydrocarbon tail. Incorporation of photoactivatable functions to cholesterol can be achieved within these three regions (Figure 34). In this section, we will focus on the synthesis of different lipid probes using the strategies described above.

3.1. Photolabeling Groups Located at the Lipid Polar Head

3.1.1. Phospholipids and Sphingolipids. As mentioned above, simple reactions under mild conditions are required to introduce the photolabeling group to the lipid. Among different reactions reported, acylation of the amine function in phosphatidylethanolamine (PE) is the most frequently used method for fast, convenient, and efficient synthesis under mild conditions. As an example, probe 18 was achieved by acylating PE with the corresponding *N*-succinimidyl activated ester in the presence of triethylamine (Figure 35).⁸¹ Similarly, coupling PE with the relevant phenyl carbamic chloride followed by subsequent deprotection and diazotization furnished probe 47 with an aryl diazonium moiety as the labeling chromophore located at the polar head (Figure 36).⁹⁷ The synthesis of GPI-

Table 2. Photoactivatable Phospholipid Probes

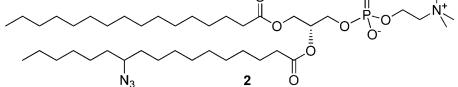
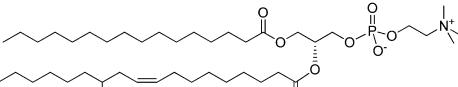
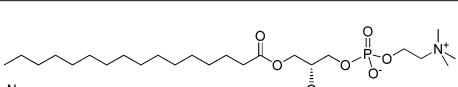
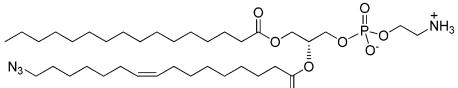
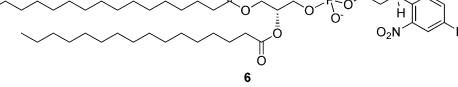
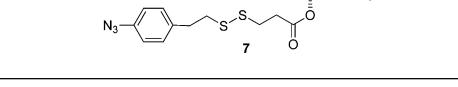
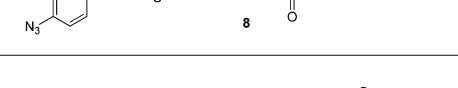
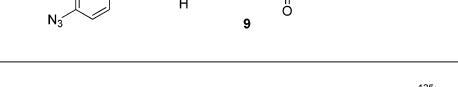
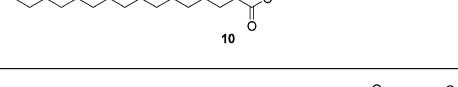
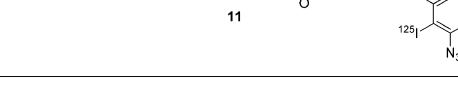
Photoactivatable lipids	References
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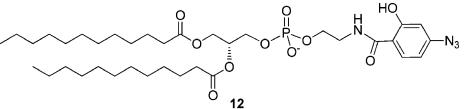
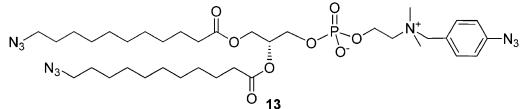
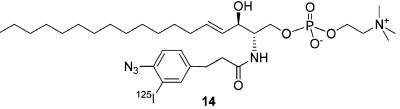
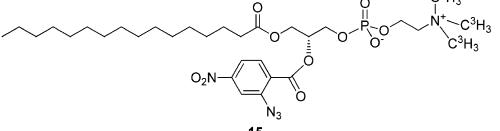
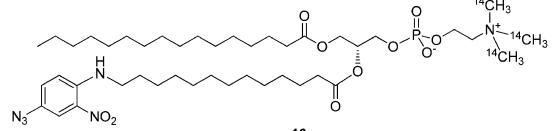
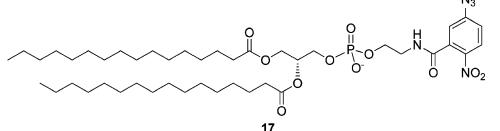
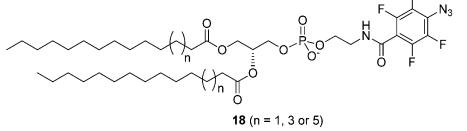
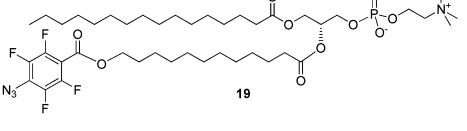
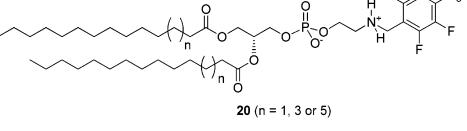
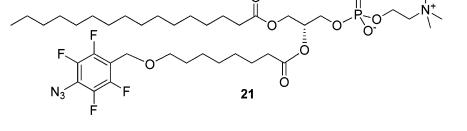
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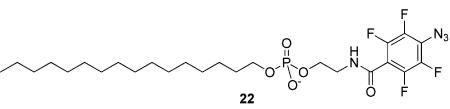
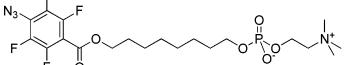
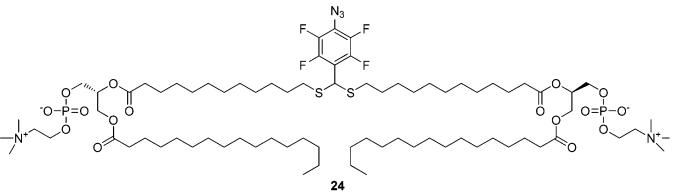
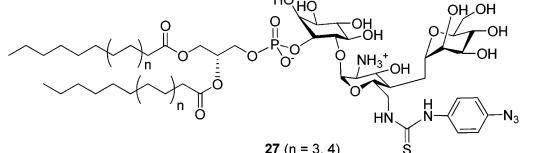
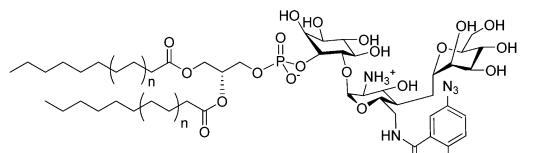
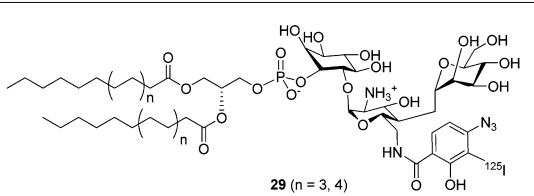
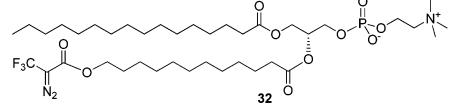
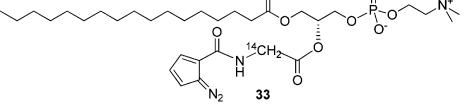
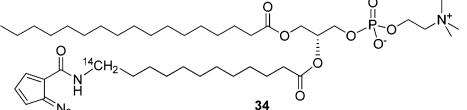
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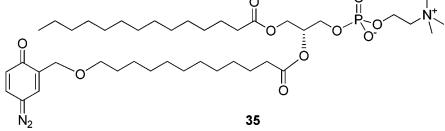
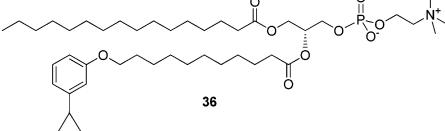
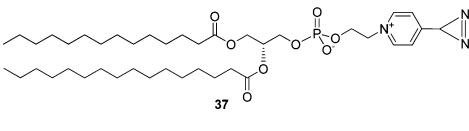
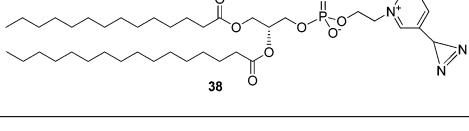
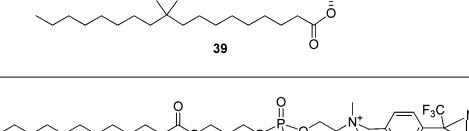
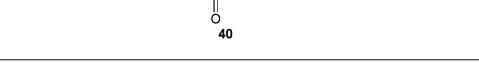
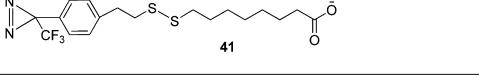
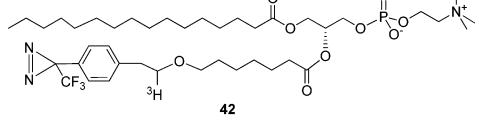
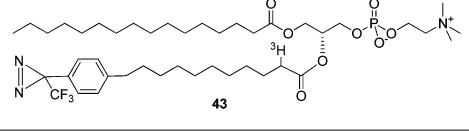
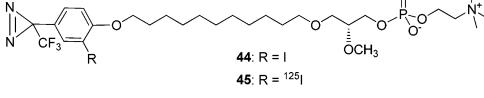
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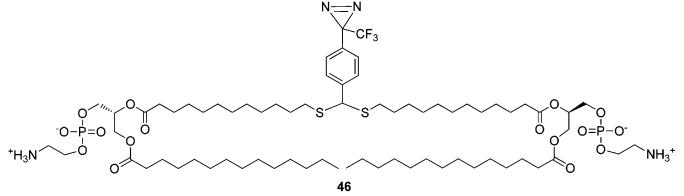
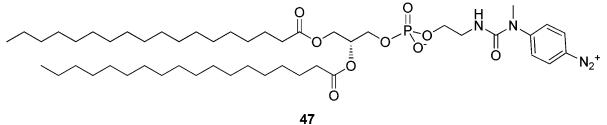
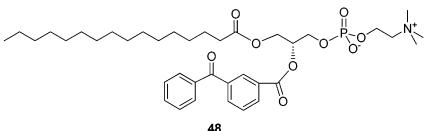
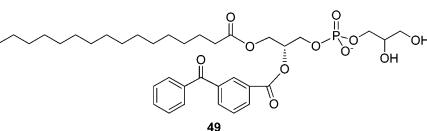
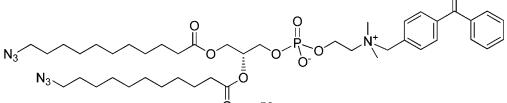
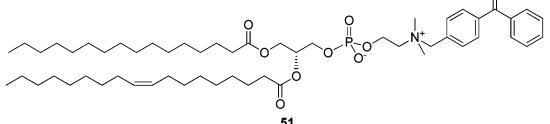
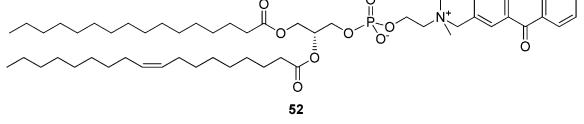
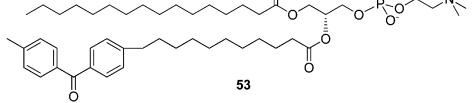
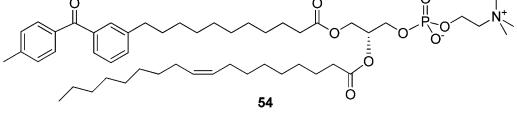
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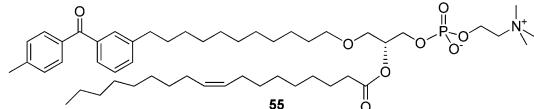
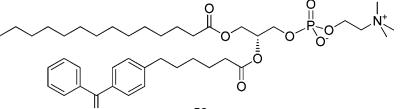
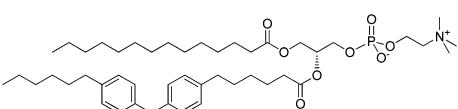
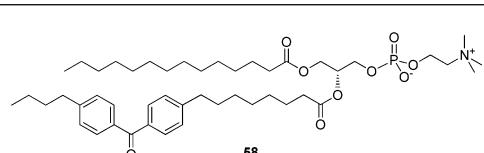
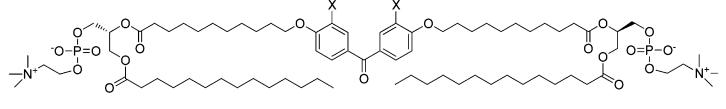
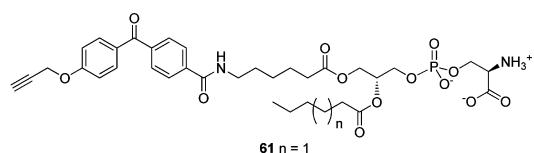
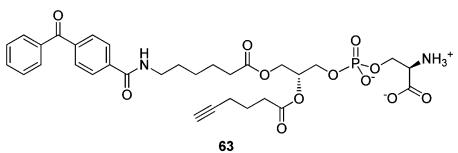
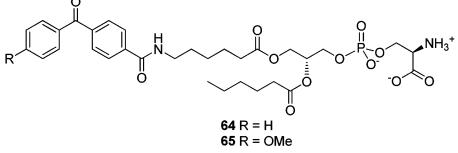
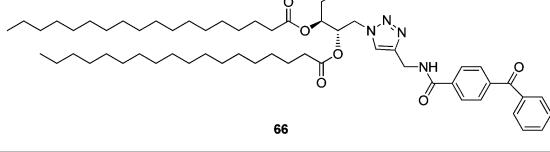
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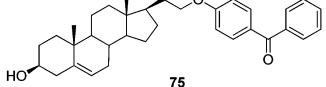
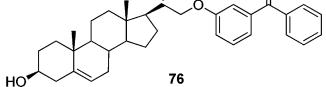
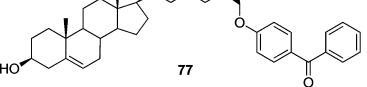
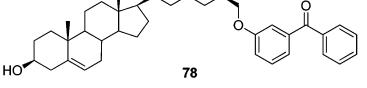
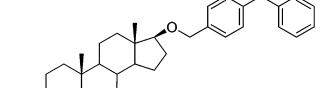
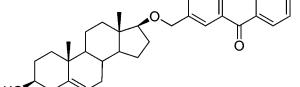
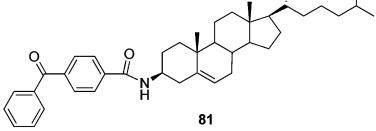
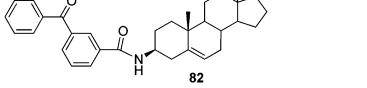
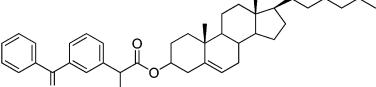
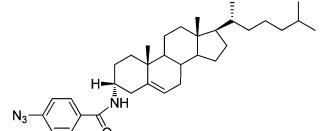
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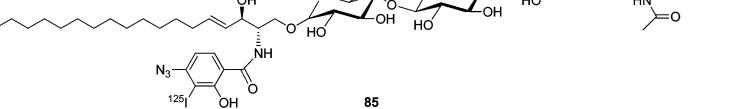
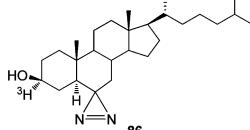
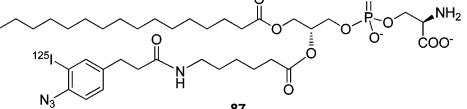
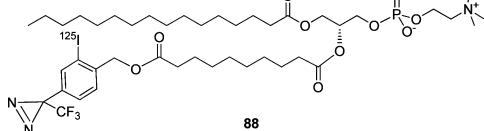
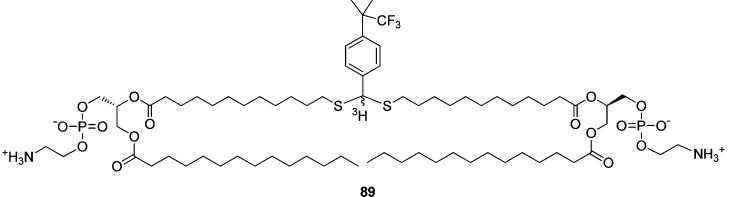
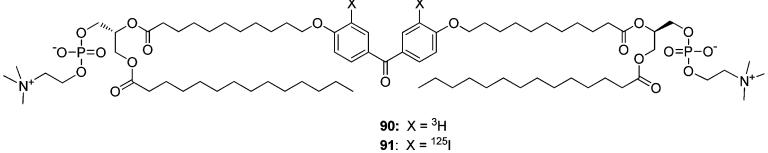
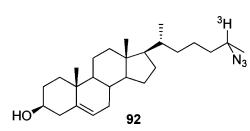
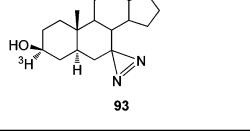
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	50, 232
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	143
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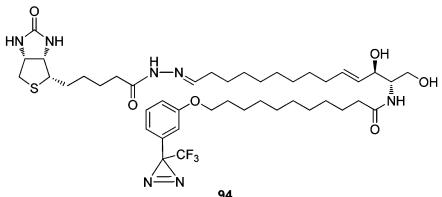
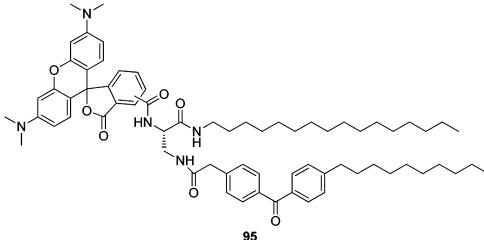
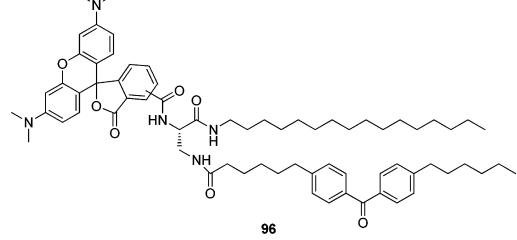
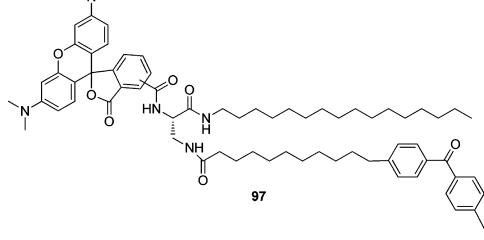
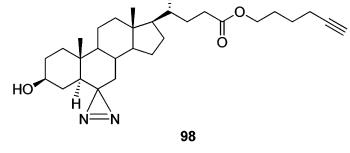
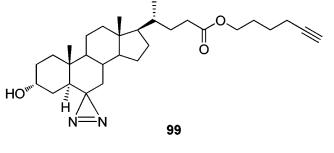
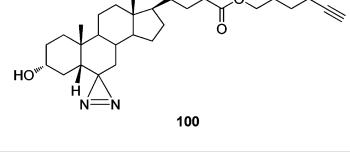
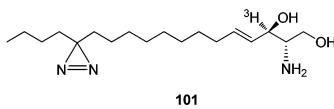
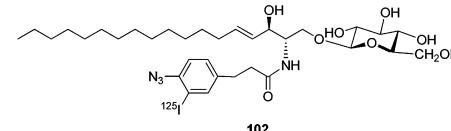
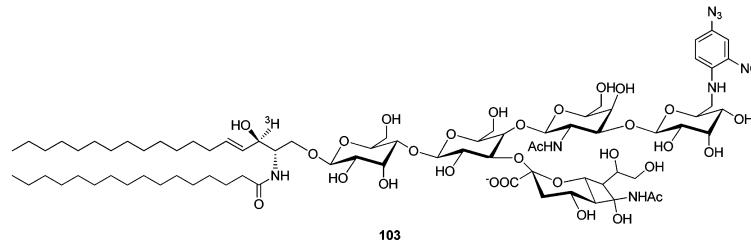
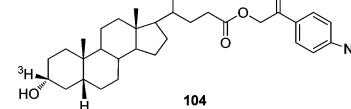
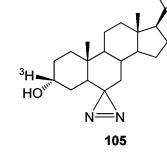
Photoactivatable lipids	References
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	156
	156
	156
	193
	193
	193

Table 2. continued

Photoactivatable lipids	References
	202, 203
	65
	204
	221
	255

based photoactivatable probe **28** started with the glycolipid analogue **26** (Figure 37), which contains the amine functionality enabling easy introduction of the photoactivatable group via a simple and efficient amidation.⁹²

Although acylation of PE is widely used, the amide linkage abolishes the positive charge of the amino group at the polar head of PE at physiological pH, which may alter the properties and/or behavior of the resulting lipid probes in biomembranes. To retain an amine function at the polar head, Xia et al. constructed probe **20** via the reductive amination of 4-azido-tetrafluorobenzaldehyde with the corresponding PE in the presence of NaBH(OAc)₃ (Figure 38).⁸³ This probe maintained the protonation ability of an amine function, highlighting its particularity and potential usefulness in investigating pH-dependent biological events in biomembranes.^{84,85}

With the same aim of mimicking and retaining the positive charge of the choline moiety at the polar head of natural phosphatidylcholine (PC), Wang et al. synthesized probes **51** and **52** (Figure 39), each possessing quaternary ammonium functions.¹¹⁵ Their synthesis was achieved via first demethylation of the commercially available PC with DABCO followed by alkylation of the resulting tertiary amine product with the corresponding bromomethylbenzophenone (Figure 39).

3.1.2. Sterols. The polar head of sterols contains a hydroxyl group, which is an ideal position to introduce photoactive chromophore when constructing cholesterol probes. Many synthesis methods involve the introduction of an amine function at this position first to allow an easier chemical importation of photoactivatable functions under milder conditions. Compound **81** (Figure 40) is such an example, where the azido group was first introduced and then transformed to amine before being subjected to acylation to yield the desired benzophenone-containing probe.¹²⁶ Another and similar example is the synthesis of probe **84** (Figure 41), which was developed by Corbin et al. to study *Torpedo californica* acetylcholine receptor.¹²⁸ It was prepared by coupling 3-aminocholest-5-ene and 4-azido-salicylic acid using dicyclohexylcarbodiimide (DCC), followed by chloramine T iodination.¹²⁸

3.2. Photolabeling Groups Located in the Hydrophobic Part of Lipids

As compared to the probes bearing a photolabeling group at the polar head, introduction of a chromophore at the hydrophobic part of the lipid can be accomplished via more varied conjugation methods. Concerning phospholipid or sphingolipid probes, modified fatty acyl chains containing photolabeling groups are often appended at the *sn*-2 position,

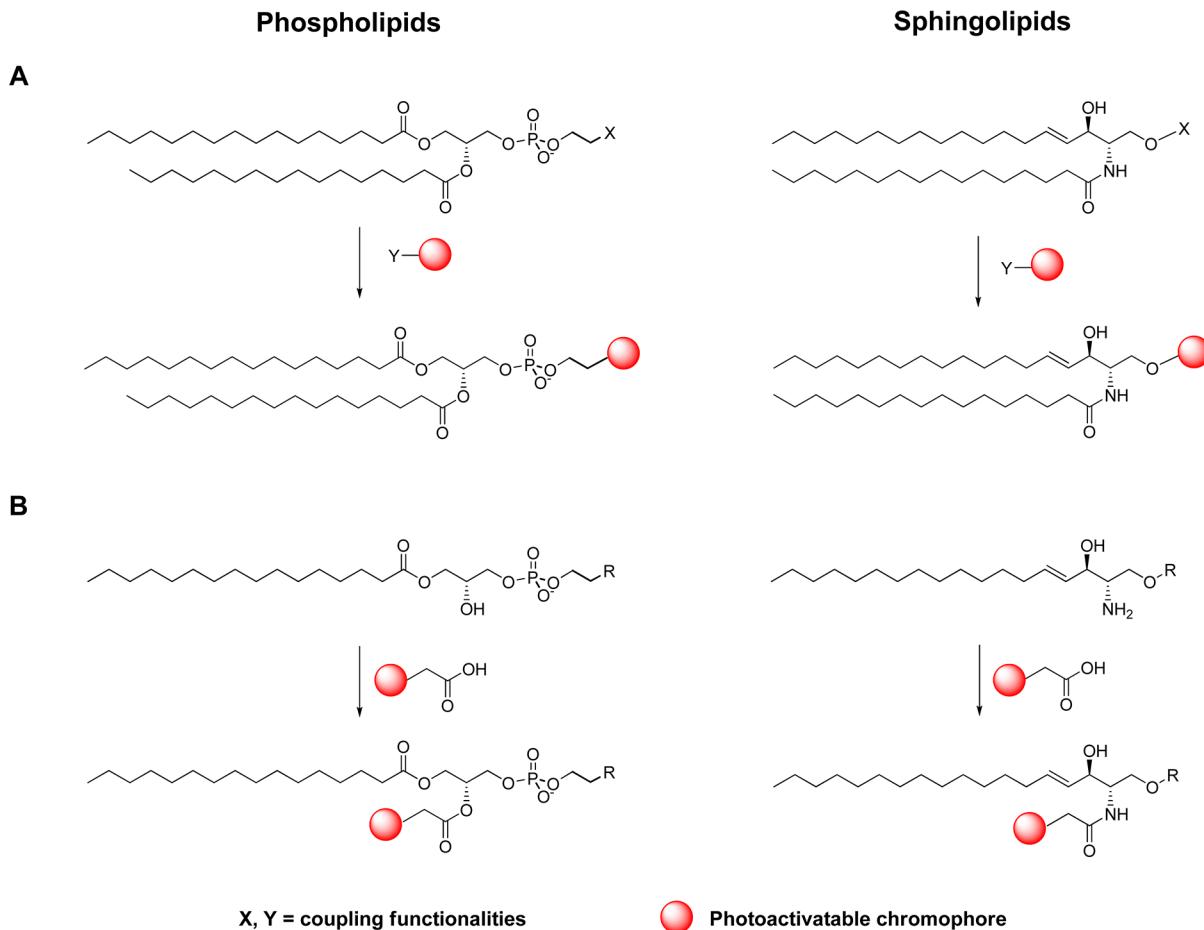


Figure 33. Strategies for synthesizing phospholipid and sphingolipid probes via modification (A) at the polar head and (B) in the fatty acyl chain, respectively.

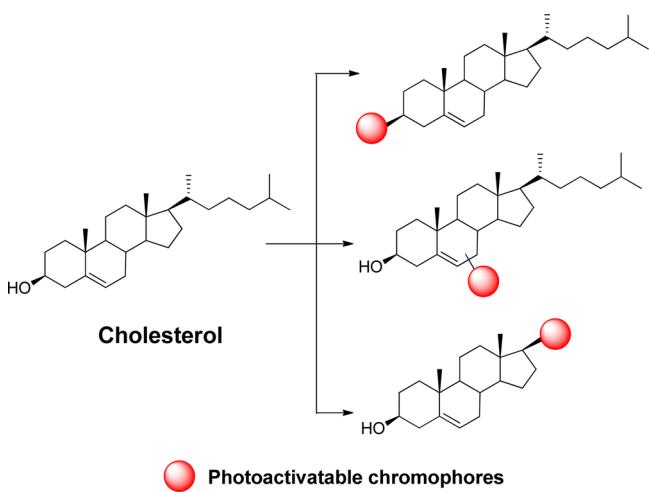


Figure 34. Synthesis of photoactivatable cholesterol probes via introduction of chromophore at the hydroxyl group position, on the fused ring skeleton, and within the hydrocarbon tail, respectively.

while diverse and specific reactions can be adopted for preparing cholesterol probes. We will not present an exhaustive list for all of the synthetic methods, but rather highlight the different variants for the probes with well-established examples.

3.2.1. Phospholipids and Sphingolipids. The synthesis of the phospholipid probe **15** (Figure 42) represents a classical case. This probe was achieved by the direct coupling of the 2-

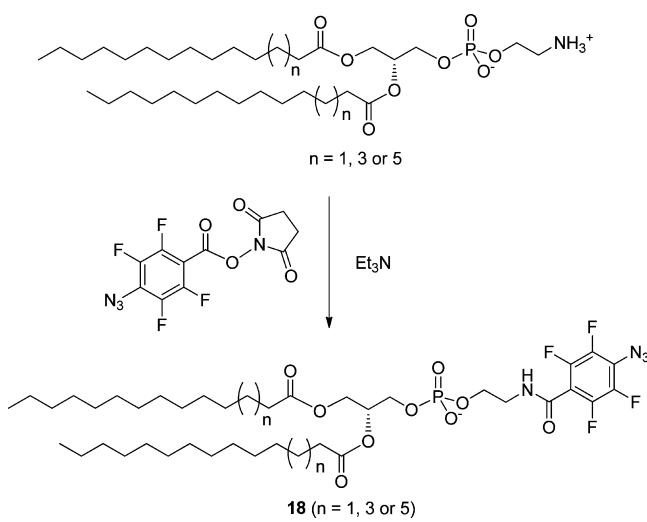
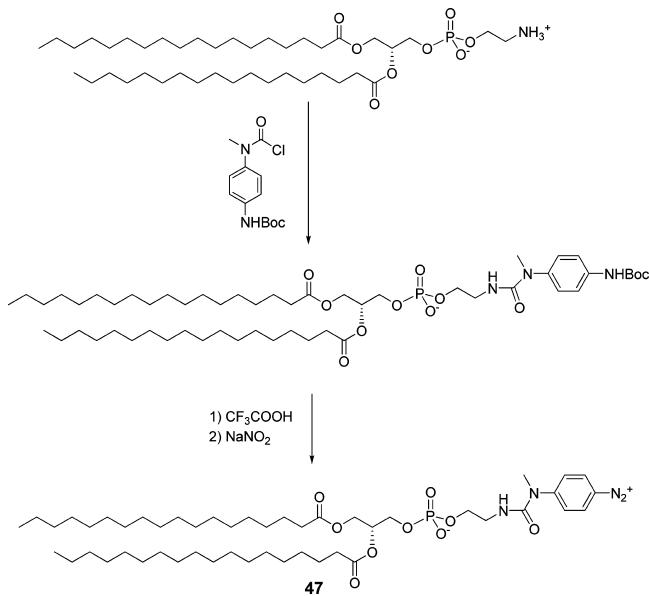
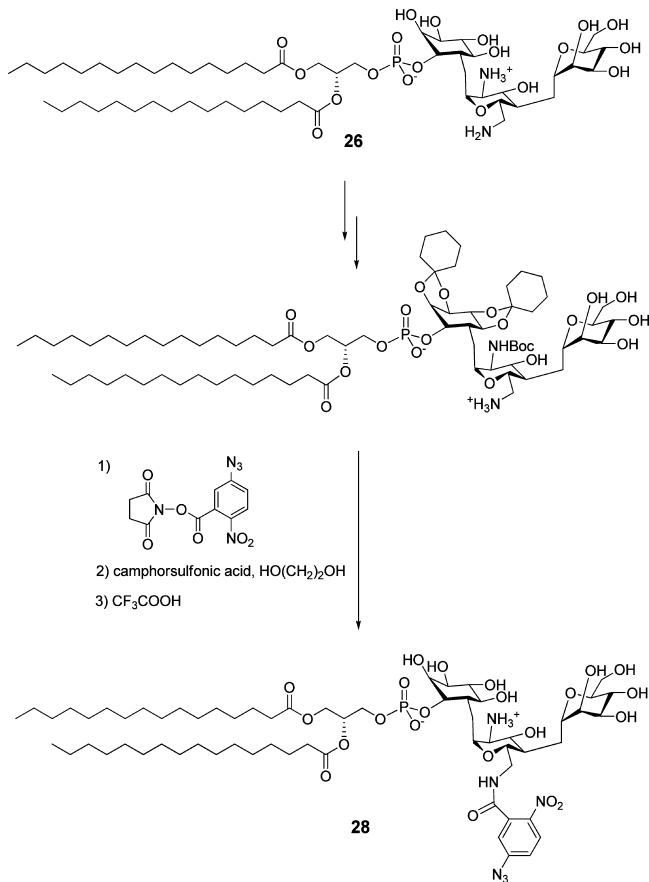


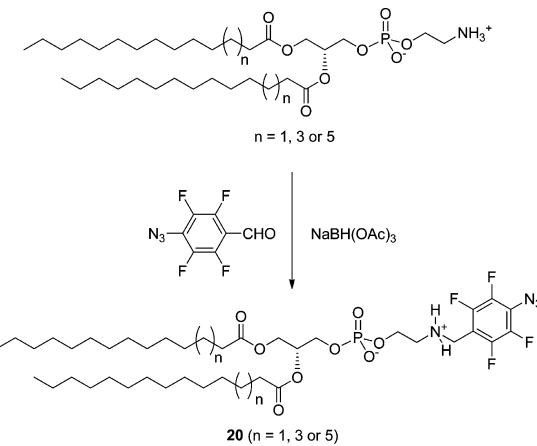
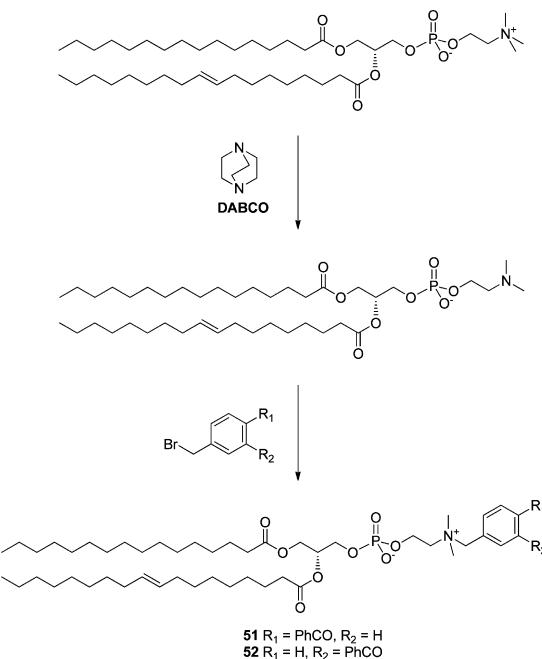
Figure 35. Synthesis of the lipid probe **18**.⁸¹

azido-4-nitrobenzoyl chromophore to lyso-PC.^{67,129} Similarly, the benzophenone-containing probe **48** (Figure 43) was achieved by condensation of the benzophenone derived acid with lyso-phosphocholine (lyso-PC).¹¹⁵ Sphingolipids carry an amine function at the *sn*-2 position, which provides an easy means to conjugate various chromophores. This feature was exploited for the synthesis of the glycolipid probe **85** (Figure 44), which was obtained by coupling lysogloboside (Gb4SPh)

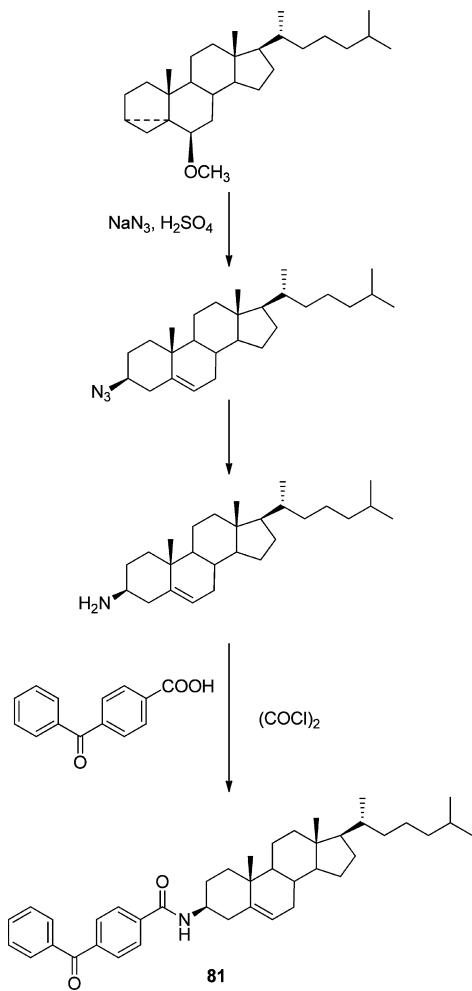
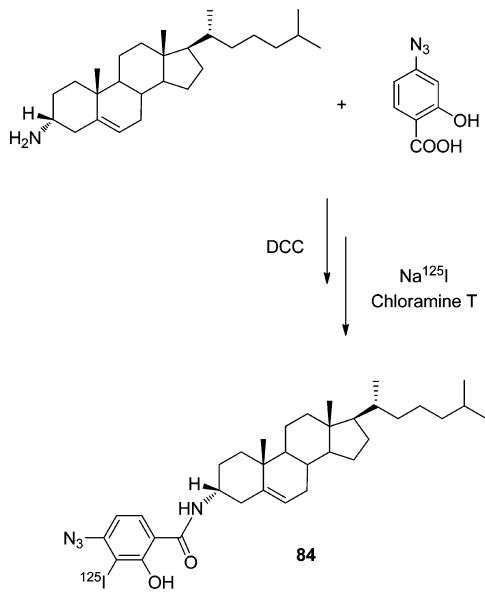
Figure 36. Synthesis of the lipid probe 47.⁹⁷Figure 37. Synthesis of the lipid probe 28.⁹²

with the active *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA), followed by subsequent radioiodination.¹³⁰

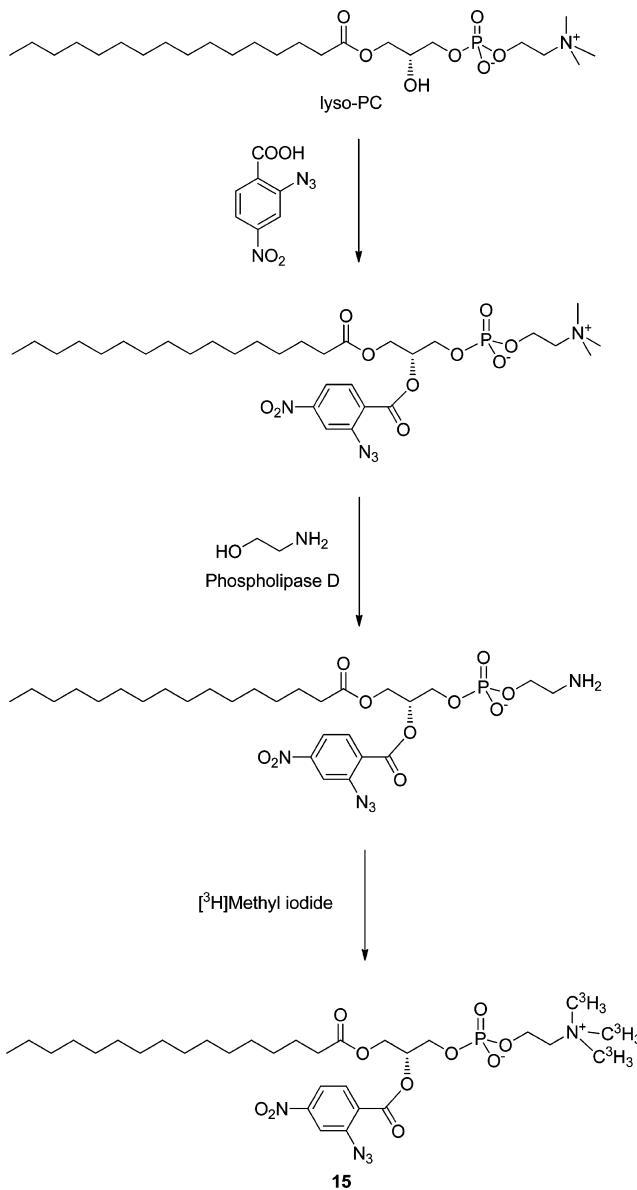
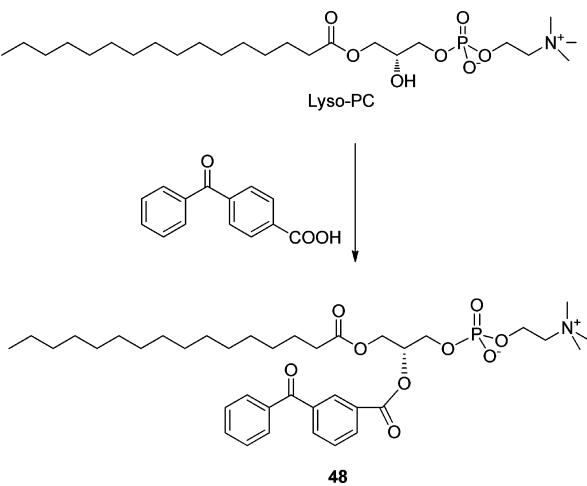
As mentioned above, lipid probes have also been developed with photochromophores located at different depths within the fatty acyl chain. Their synthesis was achieved by first constructing fatty acid derivatives carrying the labeling group at different positions along the chain and then conjugating the

Figure 38. Synthesis of compound 20.⁸³Figure 39. Synthesis of probes 51 and 52.¹¹⁵

resulting acids at the *sn*-2 position of the lipid. The approach used to introduce the photoactive group on the fatty acyl chain varies from one probe to another. For instance, while probe 16 (Figure 45)¹²⁹ probe 19 (Figure 46) has the aryl azido group introduced via amine conjugation,⁸² the synthesis of which was started from ω -hydroxy acid and followed with a series of transformations including coupling and deprotection to yield the final product. Alternatively, the chromophore can also be linked to the fatty acyl chain via an ether bond such as that used to construct probes 21 (Figure 47)⁸³ and 35 (Figure 48).⁹⁷ The advantages of ether linkage to import the chromophore are related to the relative ease of synthesis, its high stability to both chemical and enzymatic reactions, in addition to its small size and thus reduced structural diversion from natural phospholipids. However, the synthesis of such probes represents a significant challenge because the photoactivatable chromophores are often not very stable under the harsh conditions required for ether bond formation, and only a few limited reactions can be employed

Figure 40. Synthesis of the cholesterol probe 81.¹²⁶Figure 41. Synthesis of probe 84.¹²⁸

with mild conditions; this can sometimes lead to rather moderate or low yields. It is worth mentioning that any polar linker, even an ether bond, might alter the partitioning of the chain embedded in the apolar environment within the bilayer.

Figure 42. Synthesis of probe 15.^{67,129}Figure 43. Synthesis of the lipid probe 48.¹¹⁵

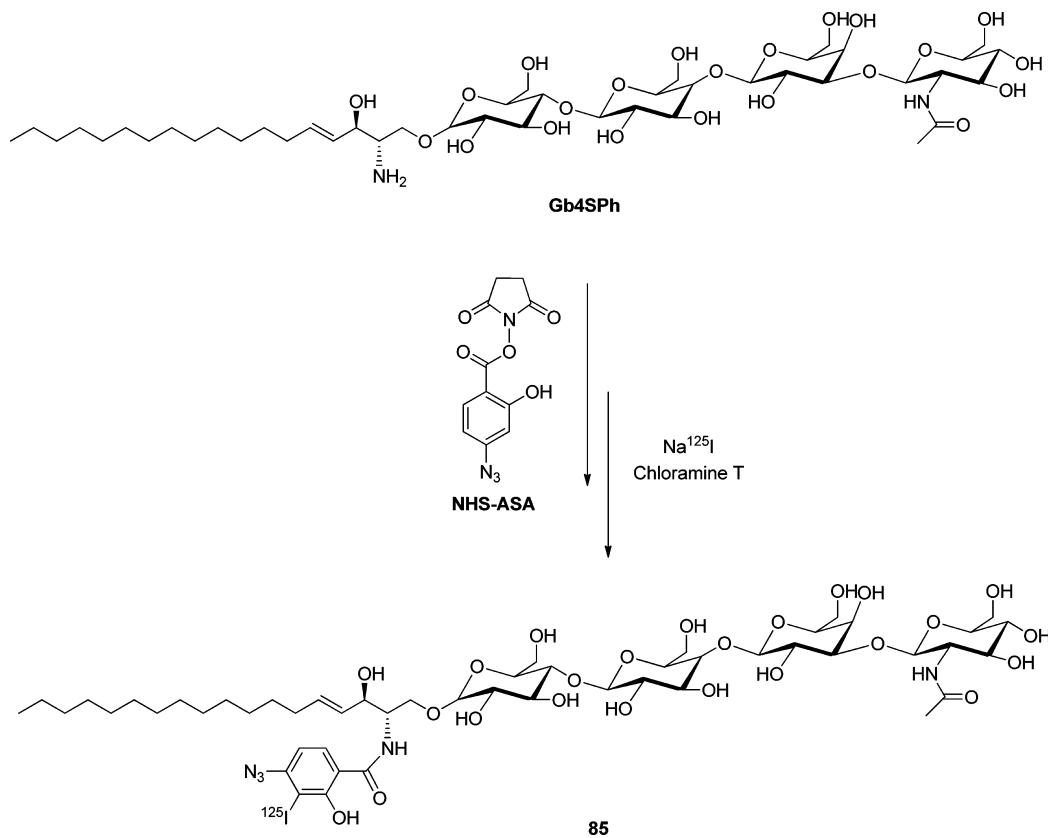


Figure 44. Synthesis of the glycosphingolipid probe 85.¹³⁰

To mimic the aliphatic chain as much as possible, photolabeling groups can also be attached to the fatty acyl chain through an alkyl linkage as shown by probe 53 in Figure 49.¹¹⁵

Incorporation of the photolabeling chromophore into bola-lipid probes usually occurs at the middle of the bipolar acid. Delfino et al. developed probe 46 (Figure 50), which has the (trifluoromethyl)phenyldiazirine chromophore introduced via the thioacetal function at the central point of the bola-lipid.⁵⁰ This synthetic strategy has recently been used by Xia et al. to prepare 24 (Figure 51) in which a tetrafluorophenylazido group is the photolabeling moiety.^{88,89} Moreover, a benzophenone chromophore was introduced at the center of the bipolar acid via two ether linkages to furnish the bola-lipid 59 (Figure 52).¹¹⁷

3.2.2. Sterols. Concerning sterols, various probes have been developed by attaching the photoactive chromophore to the hydrophobic region of either the fused rings or the tail. Their synthesis has varied from one case to another. As examples, we have shown in Figure 53 the synthesis of the two probes 76¹²⁶ and 86,¹⁰³ respectively, representing the chemical modification on the hydrocarbon tail and on the fused hydrocarbon ring part of the cholesterol.

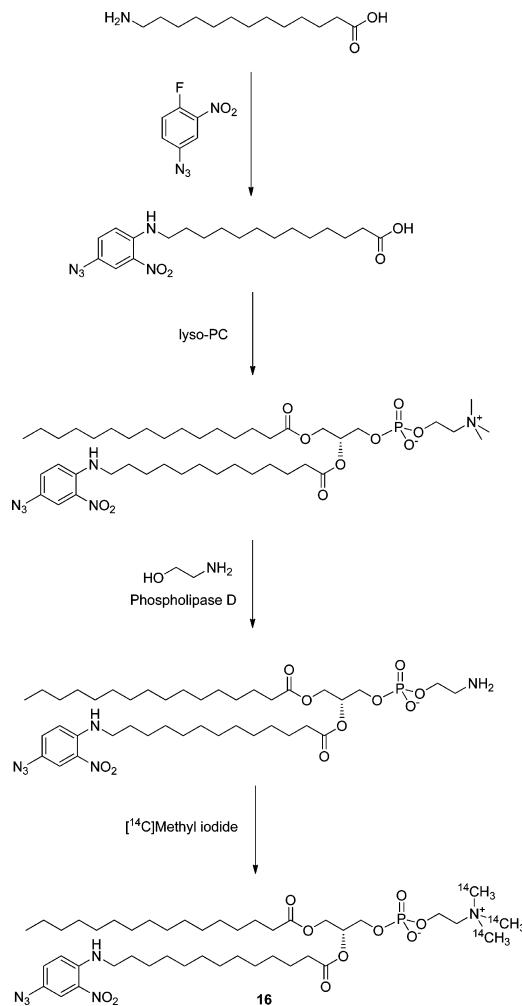
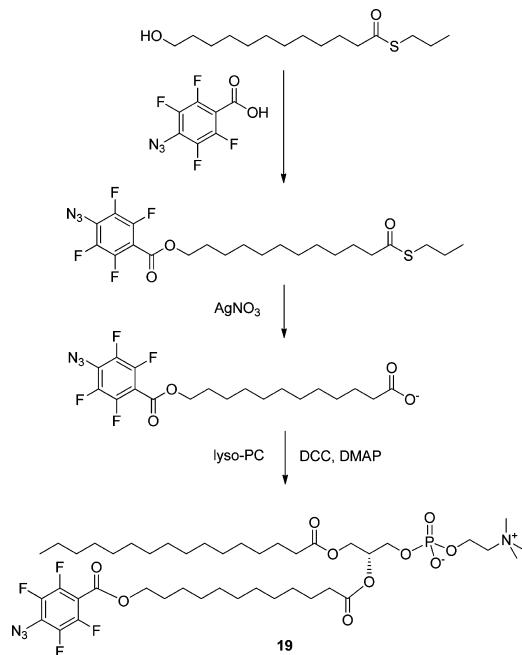
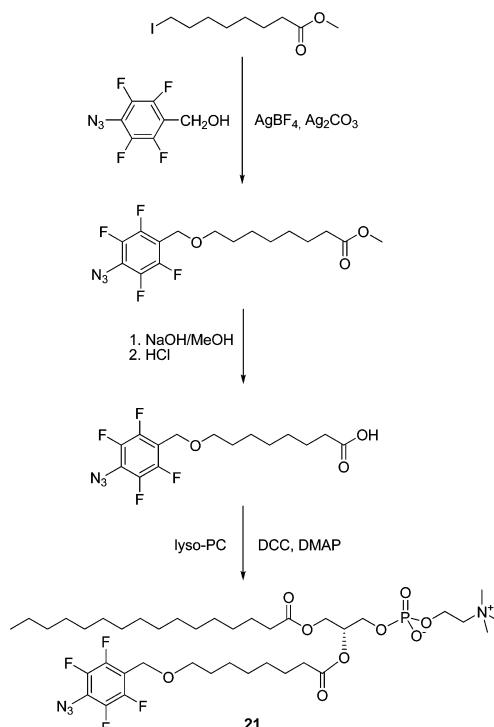
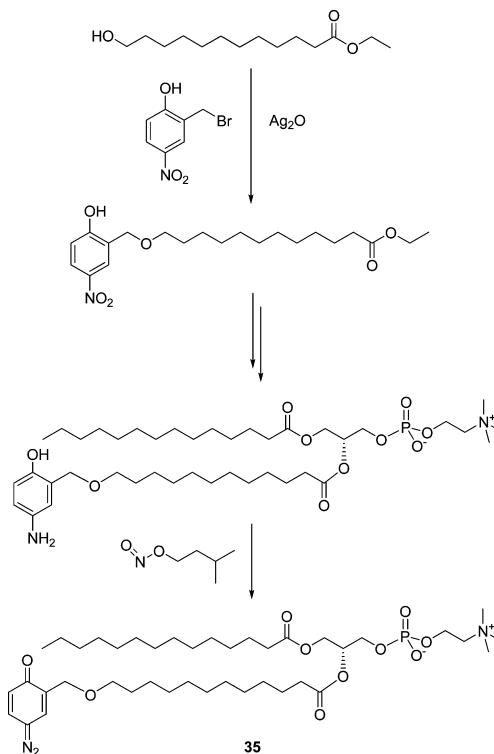
In summary, the coupling of various labeling groups to different positions on the lipid skeleton has already offered a diversity of photoactivatable lipid probes and provided valuable tools for photoaffinity labeling studies of specific regions of biomembranes. Nevertheless, scientists are currently striving toward enriching the choice of probes available and improving their efficiency to meet more specific requirements.

4. TECHNICAL ISSUES IN THE PHOTOLABELING PROCESS

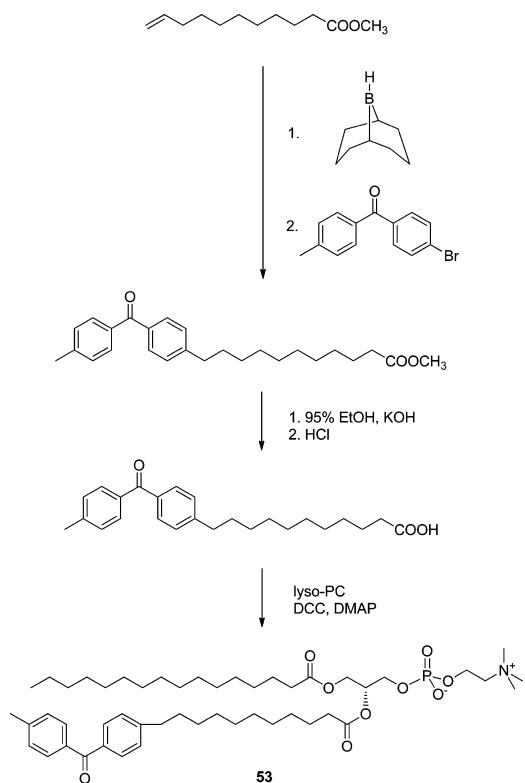
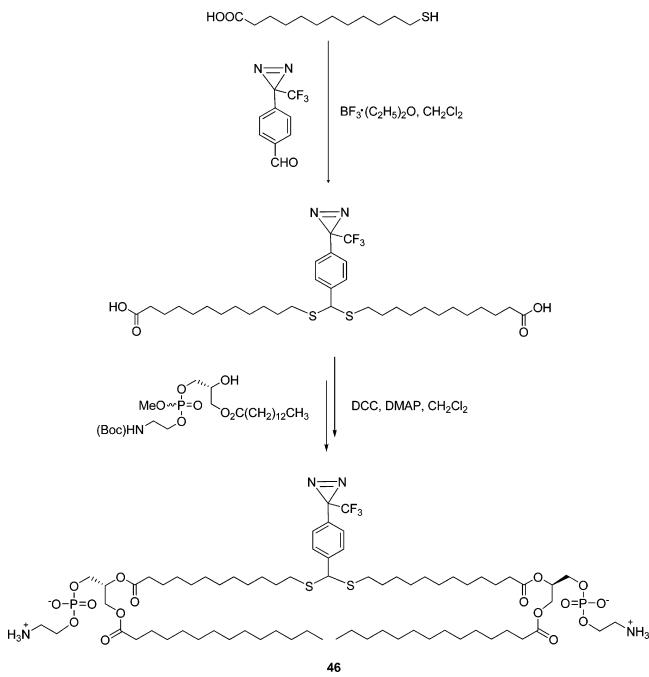
4.1. Incorporation of Lipid Probes

After their synthesis, competent lipid probes are subjected to photoirradiation for the required labeling studies in biomembranes. Prior to photoirradiation, it is important to incorporate the photoactivatable lipid probes into the model membranes or biological membranes. Usually a very low concentration of the lipid probes (5 mol % of the total lipid composition),⁶⁰ often below the critical micellar concentration, is used in photolabeling studies. Even though lipid probes can form micelles in water, exchange between the micelles and membrane structures can still occur, permitting the lipid probe to reach and insert into the membrane. So far, two major ways have been used to reconstitute the photoactivatable lipid probes into membranes according to the experimental purposes and the labeling targets.

(1) The first is direct mixing of the lipid probes with membranes, for which usually the membranes are ready for the labeling experiment and can be incorporated with the lipid probes directly. To achieve the photolabeling assay of inner mitochondrial membrane vesicles (IMV), Gubbens et al. mixed the lipid probes with IMV directly and incubated the mixture at 37 °C prior to UV irradiation.⁶⁴ Hamouda et al. performed a similar procedure to study the *Torpedo* nicotinic acetylcholine receptor (AChR) in which they first prepared freshly nAChR-enriched membranes and then mixed the membrane aliquots with the photoactivatable cholesterol probe 86 (Figure 53) in the presence or absence of carbamylcholine at room temperature for 2 h.¹³¹ Corbin et al.¹²⁸ also used a similar method to identify the cholesterol binding domain in AChR using probe 84 (Figure 41).

**Figure 45.** Synthesis of probe 16.¹²⁹**Figure 46.** Synthesis of the lipid probe 19.⁸²**Figure 47.** Synthesis of the lipid probe 21.⁸³**Figure 48.** Synthesis of probe 35.⁹⁷

(2) The second is formation of vesicles or liposomes using the lipid probes. Many experiments are also performed in vesicles or liposomes formed with natural lipids together with the photoactivatable lipid probes. Usually small unilamellar vesicles (SUVs) are prepared with bath sonication, and large unilamellar vesicles (LUVs) are obtained using either ether injection or high-pressure extrusion through polycarbonate

Figure 49. Synthesis of probe 53.¹¹⁵Figure 50. Synthesis of the bola-lipid probe 46.⁵⁰

filters. Because the lipid probes are ready to incorporate into vesicles, they can be simply mixed with the other membrane constituent lipids at the beginning of the preparation. This method represents a popular way of preparing the model membranes for photolabeling study. In their study on glycophorin A (GPA) in membrane, Ogawa et al. established different reconstitution methods for proteoliposomes composed of phospholipids, cholesterol, photoactivatable probes, and proteins, such as (1) mild sonication followed by extrusion,

(2) sonication, and (3) detergent solubilization and dilution.¹³² Their comparative studies on the reconstitution of proteoliposomes (composition of phospholipids, cholesterol, probe, and protein) showed that in the absence of GPA, sonication resulted in liposomes, in which cholesterol was well incorporated. However, these liposomes were not competent for further preparation of proteoliposomes, as most of the GPA could not be readily incorporated. On the contrary, proteoliposomes prepared by mild sonication followed by extrusion comprised lower cholesterol concentration in the membrane fraction. The best results were obtained by first mixing natural lipids with the lipid probes to form a lipid film prior to sonication, after which GPA was added, with the detergent octylglucoside as a supplement to help the solubilization and dilution. Gubbens et al. also tried the preparation of LUVs using natural lipid DOPC and DOPG together with 4 mol % of photoactivatable probe.⁶⁴ The obtained vesicles were incubated with cytochrome *c* followed by a subsequent UV photoactivation allowing quantitation of the amount of bound cytochrome *c* from the cross-linking experiments.

4.2. Analysis of Photo-Cross-Linking Products

The next challenging step following the photolabeling process is to analyze and identify the labeled lipids and membrane proteins. The high hydrophobicity of membrane proteins (especially integral proteins) usually complicates the identification and isolation process and hence hampers the subsequent analysis.¹³³ Over the years, various tags such as radioactive isotopes, biotin, fluorescent tags, or fluorour labels have been attached to the lipid probes with the aim of further facilitating the identification and analysis of the photo-cross-linking products resulting from the labeling process. In the early years, electrophoresis based on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE)¹³⁴ and high performance liquid chromatography (HPLC)¹³⁵ were the most commonly used methods for protein separation. Later, HPLC coupled with mass spectrometry (MS) emerged as the preferred method due to its remarkable superiority with regards to speed, sensitivity, resolution, and precision.^{36,136,137} A brief description of the different tags and methods for analyzing labeled products following the photolabeling experiment will be presented below.

4.2.1. Tags for Photoadduct Analysis. To facilitate the protein separation and analysis, conjugation of bioorthogonal tags has been exploited to expedite the analysis of labeling products.¹³⁸ Today, radioactive isotopes, biotin, fluorescent chromophores, or fluorour labels represent the most frequently used bioorthogonal tags incorporated within the photo-activatable lipid probes. We present below the use of these tags in photolabeling studies of biomembranes.

4.2.1.1. Radioactive Isotope Tags. Isotope labeling is the earliest, yet still the most sensitive method of tracking trace amounts of labeled proteins or other molecules.^{139,140} In addition, incorporation of the isotope element does not alter the structural or functional properties of the parent molecule. The most frequently used isotopes in photolabeling are radioactive isotopes (¹⁴C, ¹²⁵I, ³H, etc.) with the stable isotopes (¹³C, ²H, etc.) only being used in certain situations. This is simply because the radioactive isotopes (¹⁴C, ¹²⁵I, ³H, etc.) are relatively accessible and have much higher sensitivity, specificity, and accuracy for detection and quantification.

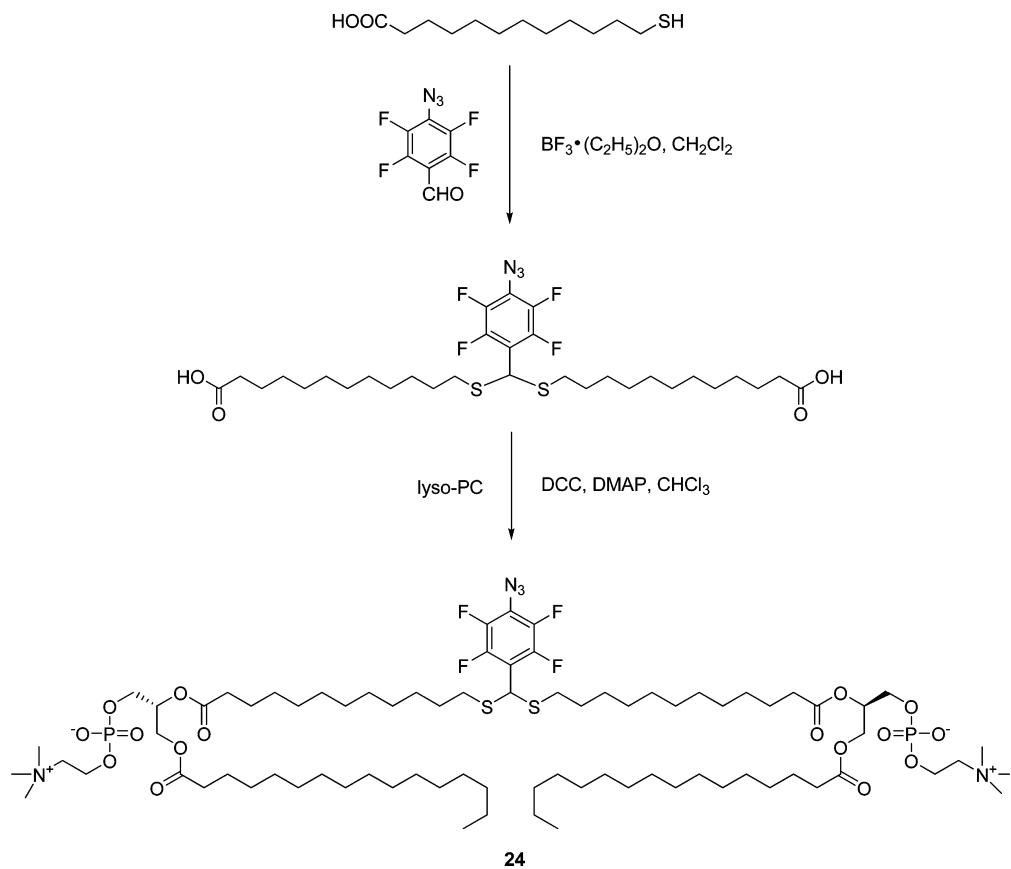


Figure 51. Synthesis of the bola-lipid probe 24.^{88,89}

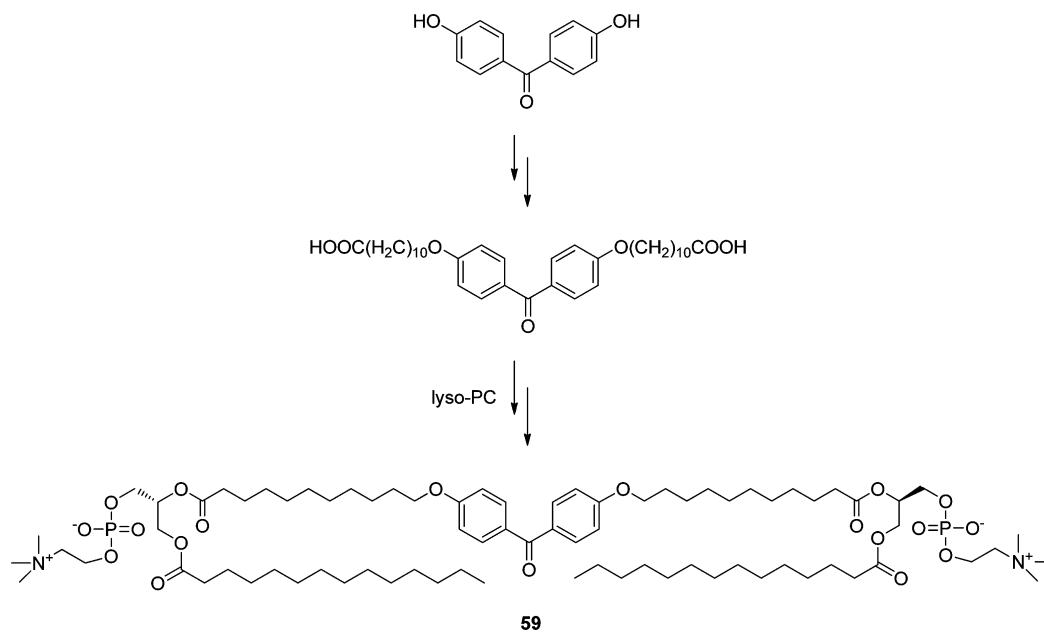


Figure 52. Synthesis of the bola-lipid probe 59.¹¹⁷

To perform such a study, an isotope element is first introduced into a probe. When the isotope tagged probe has cross-linked with the target protein, SDS-PAGE and/or HPLC separation coupled with autoradiographic identification¹⁴¹ of labeled proteins with subsequent MS analysis can provide instrumental and insightful proteomics information such as the

identification, localization, distribution, and transformation of the isotope labeled molecule.

Numerous lipid probes with isotope tags have been developed, such as 10 (Figure 9),⁶² 33,34 (Figure 18),⁹⁶ 45 (Figure 22),¹⁰⁶ 60 (Figure 29)¹¹⁷ mentioned above, as well as 87 and 88 in Figure 54.^{61,142} The radioactive isotopes are usually attached within the chromophores, thereby imparting

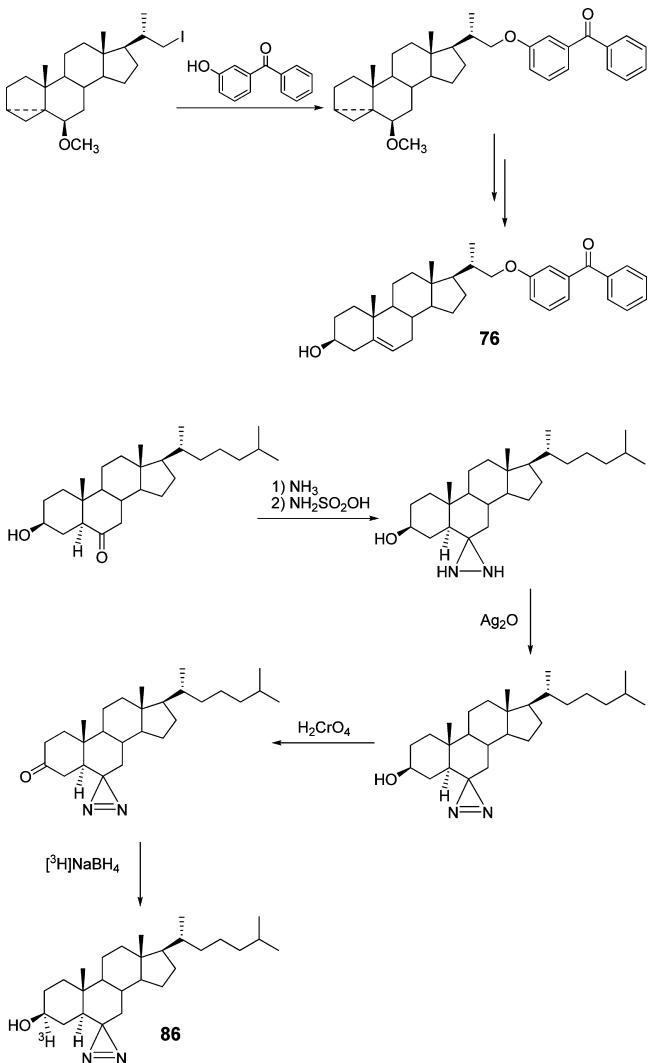


Figure 53. Synthesis of the photoactivatable cholesterol probes **76** and **86**.^{103,126}

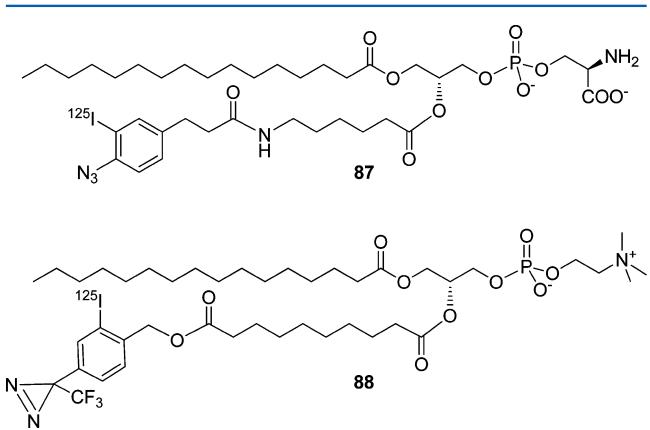


Figure 54. Phosphatidylserine probe **87** and phosphatidylcholine probe **88** containing radioactive tags.^{61,142}

the photolabeling region with radioactivity. The isotopes can also be located elsewhere other than within the chromophore as shown in **15** and **16** (Figure 10), which, respectively, harbor ³H and ¹⁴C in the choline head of the phospholipids.⁶⁷ Likewise, the bola-lipids **46** (Figure 23) and **59** (Figure 29) were further developed into their radioactive forms **89**, **90**, and **91** (Figure

55) with the aim of facilitating the process of analyzing the labeling products.^{50,132} In addition to the phospholipid probes, glycolipid and cholesterol probes have also been decorated with isotope tags. **29** (Figure 15)⁹² is a GPI analogue carrying ¹²⁵I at its carbohydrate part, while **85**¹³⁰ (Figure 44) harbors ¹²⁵I at the acyl chain region. The cholesterol analogues **86**¹⁰³ (Figure 53), **92**,¹⁴³ and **93**¹⁴⁴ (Figure 56) bear the ³H tag, whereas **84** (Figure 41)¹²⁸ carries ¹²⁵I in the phenylazido function. This list of isotope labeled probes is far from exhaustive and is only meant to serve as examples.

Although the use of radioactive tags has greatly contributed to and advanced the photolabeling studies for many years, increasing concerns about radioactive danger to health and environment substantiate stringent safety precautions and manipulation restrictions. Furthermore, radio contamination of the analytical equipment (HPLC, mass spectrophotometer, etc.), as well as the lack of disposal sites for radioactive waste has limited the use of radiation in research laboratories. Moreover, the instability of long-term storage and the short half-lives of the most commonly used radioisotopes necessitate that the material be freshly labeled to obtain optimal efficiency. Concerning the health and environmental risks as well as the manipulation restriction involved with using radioactive isotopes, an increasing number of alternative tags or methods have been developed aiming to facilitating postanalysis after photolabeling process.

4.2.1.2. Biotin Tag. As we mentioned above, despite isotope tags offering undeniable advantages and having made a significant contribution in multiple domains of biology, particularly protein science, their radioactivity hazard makes them a safety and environmental risk. As such, biotin tags came forth as a safe and effective alternative mainly due to biotin's exceptionally high affinity toward its binding proteins such as avidin or streptavidin,¹⁴⁵ which can be exploited to facilitate the detection and analysis of target proteins. Biotin is a water-soluble vitamin consisting of a tetrahydroimidazolone ring fused with a valeric acid-substituted tetrahydrothiophene ring (Figure 57).¹⁴⁶ Different functionalities can be appended to the biotin via the valeric acid side chain for further conjugation purposes.^{146,147} Additionally, considering the small size of biotin, biotinylation appears to produce relatively small variation. It is also worth noting that the molecules bearing biotin tags can be immobilized through interaction with avidin or streptavidin before being purified by affinity column.

The photoreactive ceramide analogue **94** (Figure 58) synthesized by Hashimoto and Hatanaka exemplifies the cointroduction of a diazirine chromophore and a biotin tag to a sphingolipid.¹⁴⁸ Later, probe **94** was used to photolabel *Pseudomonas* sp. sphingolipid ceramide N-deacylase (SCDase).¹⁴⁹ The biotin moiety in **94** was recognized by streptavidin–horseradish peroxidase (HRP) conjugate and a mouse anticeramide antibody, which allowed the direct detection of the photolabeled SCDase using chemiluminescence after Western blotting. The recognition of the bifunctional ceramide analogue **94** by the anticeramide antibody indicated that the probe has preserved the antigenic properties of natural sphingolipids.

In a recent application of the biotin–avidin technique in a photolabeling study, click chemistry based on alkyne/azide coupling was used inventively by Gubbens et al., allowing the detection, separation, and identification of the labeled targets.⁶⁴ The authors appended an azido group onto the hydrophobic terminal of the lipid probe. It is worth mentioning that the

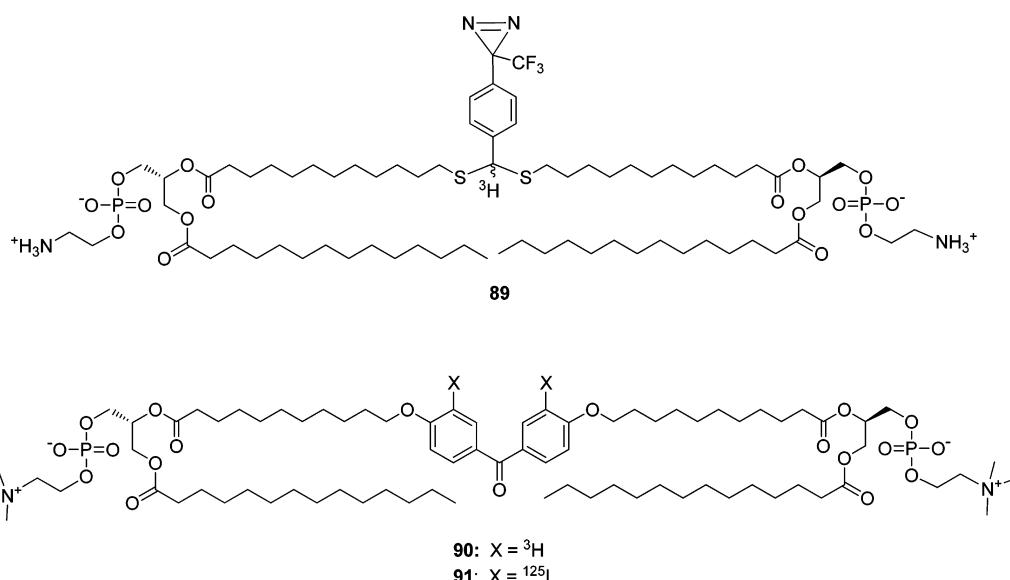


Figure 55. The bola-lipids **89**–**91** containing radioactive tags.^{50,132}

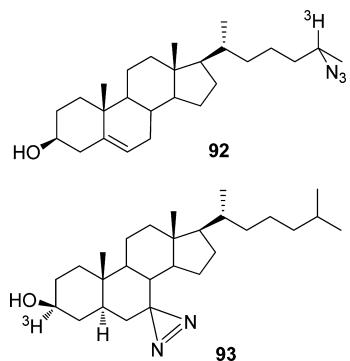


Figure 56. The cholesterol probes **92** and **93** containing radioactive tags.^{143,144}

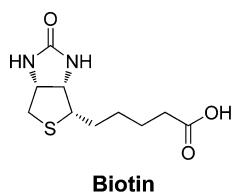


Figure 57. Structure of biotin.

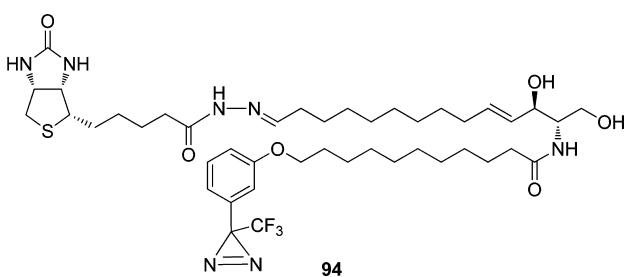


Figure 58. The bifunctional ceramide probe **94** harboring both a photoactivatable group and a biotin moiety.¹⁴⁸

azido group here had no photolabeling purpose, but rather an analytical purpose for postlabeling analysis via click chemistry. After photo-cross-linking, the azido groups present in the lipids

can undergo alkyne/azide click chemistry with the alkyne-bearing biotin conjugate. The so resulted products can be picked up via binding to a neutravidin-horseradish peroxidase (HRP) conjugate. Subsequent mass spectral analysis leads to the identification of the labeled proteins (Figure 59). This specific strategy used for the study of lipid–protein interactions in biomembranes has been recently highlighted in several reviews, and readers are referred to them for detailed examples.^{138,150}

Although the biotin-based system benefits greatly from the high affinity between biotin and avidin or streptavidin for efficient protein separation and purification in the photoaffinity labeling studies, this approach is sometimes plagued with irreversibility under physiological conditions¹⁵¹ and significant background noise due to the presence of natural biotin in living cells. Further improvements in biotin related systems to enhance their worth are therefore required, and/or the discovery of more competent tags for analytical purposes will be useful to the development in this field.

4.2.1.3. Fluorescent Tags. Aside from biotin tags, fluorescent tags constitute another commonly used means of detecting and identifying labeled products in photolabeling studies. They not only boast a high sensitivity but are also easy and practical to handle as well as being environmentally benign.^{152–155} With the aim of examining the photolabeled membrane components and exploring their corresponding structural properties, scientists have tagged fluorescent moieties to lipid probes to facilitate the analytical process. Recently, Hilbold et al. developed lipid probes **95**–**97** (Figure 60) for the photolabeling of bacteriorhodopsin.¹⁵⁶ These probes have a benzophenone chromophore on the fatty acyl chain as the photolabeling moiety and a rhodamine moiety at the polar head as the fluorescent tag. Experimental results with these probes demonstrated that the benzophenone entity could form covalently cross-linked products with members of its immediate environment within the membrane, while the rhodamine-containing headgroup allowed the sorting of the photoadducts.¹⁵⁶ The PIP_n probe **71** (Figure 31) developed by Rowland et al. also contains fluorescein in the structure, which serves for optical detection.¹²⁴ Additionally, Gubbens et al. employed click chemistry to attach a rhodamine component as

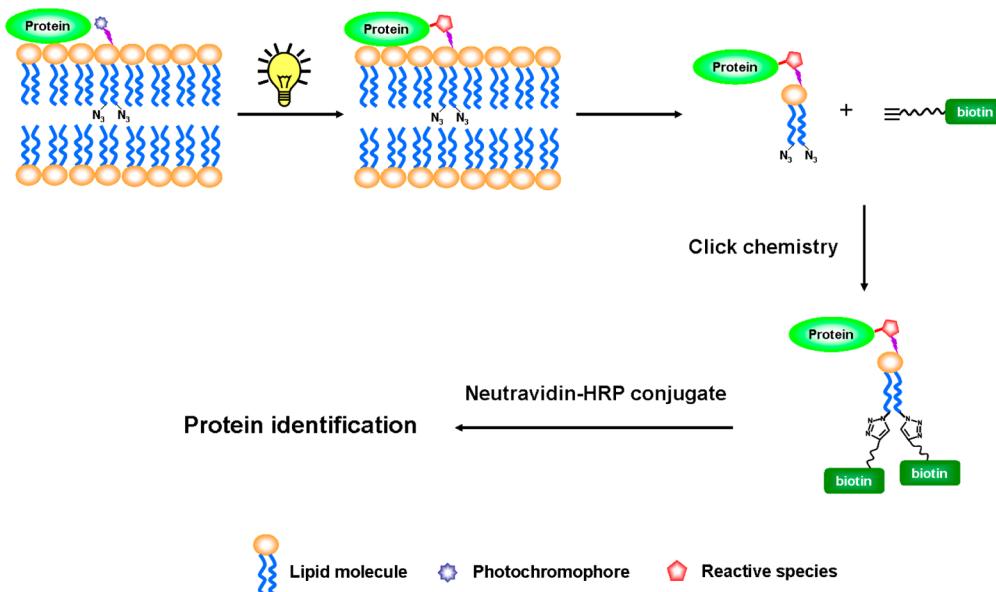


Figure 59. The photoactivatable phospholipid containing azides as fishing bait is incorporated in a biological membrane. After photo-cross-linking, alkyne/azide click chemistry is employed to selectively attach the biotin to the azido-containing lipids. Further binding with a neutravidin-horseradish peroxidase (HRP) conjugate on Western blotting leads to the identification of the labeled proteins.⁶⁴

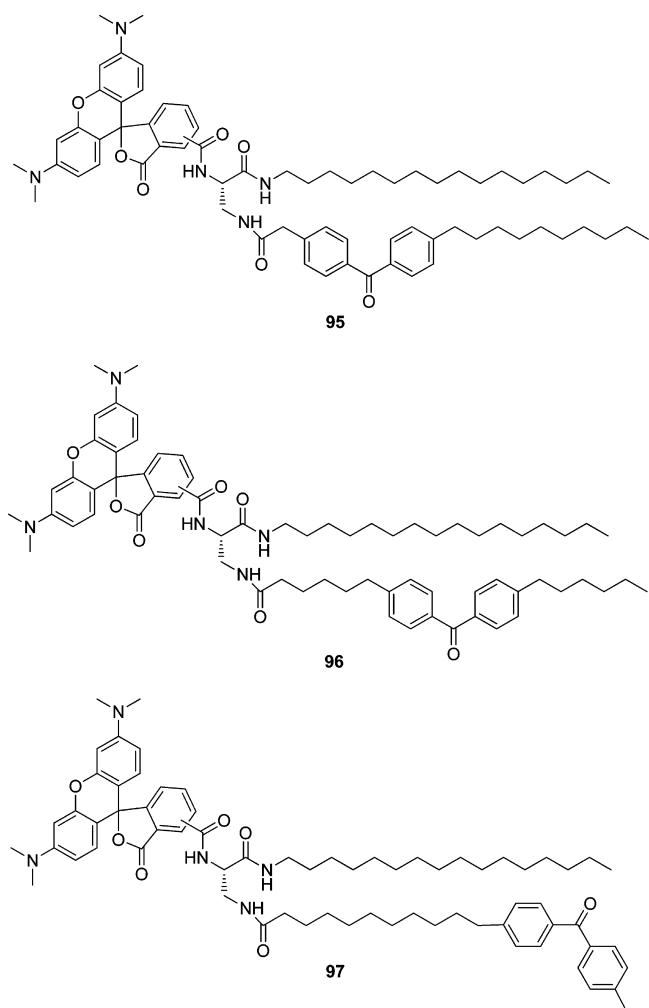


Figure 60. Photoactivatable lipid probes **95–97** carrying a fluorescent tag.¹⁵⁶

the fluorescent reporter to the lipid probes, thus helping the detection of the cross-linked products while avoiding radioactive assay.⁶⁴ In their study, after the cross-linking process, proteins were isolated from the reaction mixture and scanned for fluorescence. Cross-linking with both probes **11** (Figure 9) and **50** (Figure 27) yielded multiple fluorescent protein bands, most of which showed unaltered intensity by the carbonate wash, suggesting that the probes cross-linked membrane proteins and that the labeled proteins were thereby rendered carbonate wash-resistant by the covalent linkage to the lipid probes.

4.2.1.4. Fluorous Tag. Recently, with the development of fluorous solid-phase extraction (FSPE) and fluorous chromatography for quick separation of fluorinated molecules,^{157–159} fluorous tagged probes appear tempting for use in photolabeling studies.^{160–162} Fluorous tags have high chemical stability and minimal steric effect on their parent molecules. Moreover, FSPE and fluorous chromatography are able to specifically isolate fluorinated components from the mixture and hence greatly improve the efficiency of protein purification.¹⁵⁹ Fluorinated aryl azides and aryl-trifluoromethyl-diazirines can be considered for use as fluorous tagged photolabeling chromophores. The lipids **18–23** (Figure 13)^{81–83,86} and **24** (Figure 14)⁸⁹ developed in our group represent such examples, as do the probes **41** (Figure 21),⁶⁰ **42,43** (Figure 21),^{104,105} **44,45** (Figure 22),¹⁰⁶ **46** (Figure 23),⁵⁰ and **88** (Figure 54),¹⁴² which contain aryl-trifluoromethyl-diazirine. Considering the current flourishing in fluorine chemistry, we anticipate the forthcoming application of these probes in photolabeling studies making use of the fluorous techniques for the analysis of photoproducts.

4.2.2. Methods for Analyzing Labeled Proteins and Peptides. **4.2.2.1. SDS-PAGE.** In the early photolabeling studies, the labeled proteins were mainly identified and separated using SDS-PAGE provided that a detectable moiety was present in the lipid probes. This technique, still widely used in biochemistry and molecular biology, allows the separation of proteins, peptides, or other macromolecules based on their

electrophoretic mobility, and the determination of the relative abundance or distribution of proteins according to their signal intensity.^{134,163} By using SDS-PAGE, Schroit et al. found that the phosphatidylserine probe 87 (Figure 54) preferentially labeled a peptide with a molecular weight of 30 kDa when they used this radioiodinated probe to study the human erythrocyte cell membrane.⁶¹ In the work of Janssen et al., a mitochondrial membrane protein of 70 kDa was labeled with the phosphatidylcholine probe 88 (Figure 54).^{142,164} Further isolation of this labeled protein using 2D-gel electrophoresis revealed the interaction between phosphatidylcholine and glycerol-3-phosphate dehydrogenase (Gut2p) in yeast mitochondria.¹⁴² The isolated protein was then subjected to trypsin digestion followed by subsequent MS analysis, leading to the identification of the yeast mitochondrial Gut2p. Since then, many photolabeling studies with lipid probes have used similar approaches to analyze the labeled protein. A more recent paradigm using SDS-PAGE for photoproduct analysis concerned the isolation and identification of a P-type ATPase labeled by probe 88 (Figure 54),¹⁶⁵ which delivered data with which the lipid–protein stoichiometry could be determined.

4.2.2.2. HPLC/Amino Acid Sequencing. Although SDS-PAGE has frequently been applied to identify the labeled proteins after photolysis,^{50,61,102,142} it also suffers from several technical deficiencies. This technique, semiquantitative at best, can lead to the overestimation of molecular mass, is less effective for proteins with similar molecular mass, has only a modest throughput rate, and can cause protein denaturation, all of which limit its sensitivity and reproducibility. Therefore, HPLC has arisen as an attractive option offering higher sensitivity and resolution power¹⁶⁶ that favor quick and highly efficient separation of proteins and peptides as well as other biomolecules.¹³⁶ Similar to SDS-PAGE, a detection tag should be attached to the sample ready for HPLC analysis. Blanton et al. used reverse-phase HPLC to separate the probe 11 (Figure 9) labeled peptides to identify the transmembrane spanning region of the *Torpedo californica* nicotinic acetylcholine receptor (AChR).^{63,167} Corbin et al. then separated, via reverse-phase HPLC, the fragments of AChR subunits labeled with the cholesterol probe 84 (Figure 41),¹²⁸ and the subsequent sequencing successfully identified the major labeled amino acid residues including Lys330 on the α -subunit, Pro315 on the β -subunit, and Phe383 on the γ -subunit. Numerous other examples of how this approach has been exploited can be found in the literature, and as such we will not detail them all here.

4.2.2.3. HPLC/MS. Requiring only trace amounts of sample, mass spectrometry (MS) allows a high sensitive and fast analysis and precise molecular weight, and hence structural information by identifying the distinctive fragmentation patterns. As such, the advent of HPLC–MS coupling represented the start of a new era in analyzing photolabeling products with markedly improved sensitivity and reliability.^{168–172} In 2003, Leite et al. used “on-line” liquid chromatography (LC) coupled with MS/MS to determine the incorporation sites of the cholesterol probes and the corresponding labeled peptides in the open, closed, and desensitized states of AChR, respectively.¹²⁷ Gubbens et al. also carried out LC–MS/MS analysis to study the candidate proteins potentially involved in the interaction of proteins at the inner mitochondrial membranes of *Saccharomyces cerevisiae* using the phospholipid probe.¹⁷³ Later, the same research group continued to use LC-MS/MS to analyze the inner

mitochondrial membrane proteins photo-cross-linked with lipid probes possessing biotin tags with a view to achieving a more insightful analysis.⁶⁴ Their work showed that the attachment of biotin by click chemistry enabled affinity purification of cross-linked proteins by HPLC, and subsequent identification by MS. Collectively, SDS-PAGE together with HPLC–MS/MS is nowadays the method of choice for scrutinizing the cross-linking products of the photolabeling process.

We have outlined the major methods for photoadduct analysis, SDS-PAGE, HPLC, and MS, which play important roles in protein separation, purification, and identification. Combining various strategies with different analytical techniques has ensured continued progress in this field. Moreover, the incorporation of various tags into the lipid probes has facilitated the detection and identification process. The reported achievements promise great prospects for developing novel tags that will facilitate the sorting of labeled targets. We anticipate that photolabeling studies and their corresponding photoproduct analysis will continue to benefit from the fast development of new analytical techniques or new combinations of existing methods.

5. PHOTOLABELING STUDIES OF BIOMEMBRANES

Since photolabeling studies of biomembranes were initiated in the early 1970s using photoactivatable lipid probes,²⁹ progress made in this direction with lipid probe optimization has yielded very useful and critical information on biomembranes. In general, most photoactivatable lipid probes have been applied to explore the interactions of membrane proteins with lipids, their embedment within the lipid bilayer, as well as related structural and functional information with the aim of obtaining insightful knowledge on biomembranes. Here, we will give a brief overview in this regard.

5.1. Lipid–Protein Interactions

One important aspect of photolabeling studies on biomembranes is investigating lipid–protein interactions. Indeed, in biomembranes, membrane proteins are often in contact with different lipid species on which their function relies closely.^{16,18} Lipid–protein interactions play a considerable role in maintaining the structure and function of biomembranes,^{37,39} and studying such interactions is thus fundamental to achieving a better understanding of biomembranes.^{37,39} Fluorescence and electron spin resonance (ESR) methods were developed to probe lipid–protein interactions and are still regarded as being valuable for such studies.^{174,175} The advent of photoaffinity labeling however provided more direct and convincing evidence of lipid–protein interactions by virtue of covalent labeling and the consequent identification of the lipids and amino acid residues involved in the interacting regions. Over the past 40 years, various lipid probes for photolabeling studies on lipid–protein interactions have been developed. We will highlight such studies below, starting with phospholipid probes followed by cholesterol and other probes.

5.1.1. Phospholipid Probes. Photolabeling studies on the interaction between phospholipids and membrane proteins have mainly been achieved using probes bearing photoactive groups either at the polar head or in the hydrophobic region of the lipids. The first phospholipid probes 2–6 (Figure 8) were aimed at studying the hydrophobic interactions occurring within biomembranes.²⁹ Photolysis of the lipid vesicles containing these probes revealed the intermolecular cross-linking of the fatty acyl chains of these lipid probes, which

pioneered the journey toward developing photolabeling probes for investigating lipid/lipid and lipid/protein interactions.

The hydrophobic nature of membrane, especially integral proteins, was presumed to allow strong and specific interactions with phospholipids. It is worth noting that the affinity of membrane proteins differs for the various lipids. Certain lipids, known as annular lipids, may become enriched around a particular protein, thus enabling and enforcing the corresponding lipid–protein interactions (Figure 61).¹⁷⁸ To explore this

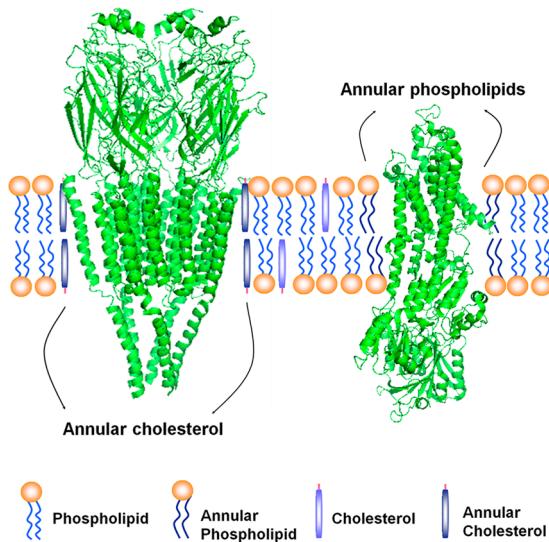


Figure 61. Annular phospholipid refers to the first molecular layer of phospholipid surrounding a cholesterol-rich domain or a protein. Annular cholesterol refers to the first molecular layer of cholesterol surrounding a protein.¹⁷⁶ The protein on the left is the *Torpedo* acetylcholine receptor,¹⁷⁸ and the one on the right is the rabbit skeletal muscle Ca²⁺-ATPase.³

issue, Giraldo et al. used ¹²⁵I-labeled PC probe 88 (Figure 54) to inspect the plasma membrane calcium pump (PMCA) and its surrounding lipids.¹⁷⁷ In their work, the PMCA was reconstituted within micelles composed of phosphatidylcholine (PC) and probe 88. They assessed the extent of hydrophobic photolabeling among the different regions of PMCA and found that 26%, 38%, and 36% of 88 interacted with the N-terminal, the C-terminal, and the central membrane regions of PMCA, respectively.¹⁷⁷ Further lipid analysis led to the conclusion that 17 ± 1 individual PC molecules were in close contact with the transmembrane surface per PMCA molecule.¹⁷⁷ This work illustrates the value of the photolabeling approach in surveying lipid–protein interactions.

The same research group also applied probe 88 (Figure 54) to evaluate the lipid–protein interactions of other membrane proteins, such as the sarcoplasmic reticulum calcium pump (SERCA) and Na⁺, K⁺-ATPase.¹⁶⁵ In the presence of a nonionic detergent, probe 88 was inserted into the mixture of dimyristoyl phosphatidylcholine (DMPC) and corresponding membrane proteins. After the photolysis, the labeled proteins were isolated by electrophoresis and the final lipid–protein stoichiometry validated by assessing the extent of the labeling reactions. Because the lipid–protein stoichiometry involves analyzing the annular lipids, the first shell of lipids in immediate contact with the membrane-embedded surface of the protein, this study therefore distinguished annular lipids from those lipids belonging to the bulk phase. As compared to the electron

paramagnetic resonance (EPR) method, the photolabeling approach using phospholipid probes described here is more sensitive and suitable for investigating lipid–protein stoichiometry because it focuses on the analysis of the membrane proteins rather than on lipid behavior to which EPR tends.¹⁶⁵

The interaction of membrane proteins with lipids has also been explored using probes containing a photoactivatable moiety at the polar head expected to selectively cross-link the proteins located at the membrane–water interface. Phospholipid probe 12 (Figure 9) is one example, which successfully labeled a series of proteins at the membrane–water interface.¹⁷³ As PC is often more abundant than PE in biomembranes, Gubbens et al. developed novel PC probes 13 (Figure 9), 40 (Figure 21), and 50 (Figure 27), which bear respectively phenylazide, trifluoromethyl diazirine, and benzophenone as the photoactivatable group at the polar head.⁶⁴ Their results showed that lipid analogues containing benzophenone and phenylazide as photoactivatable moieties can be cross-linked to specific, partially overlapping subsets of proteins, as detected by a fluorescent reporter. Meanwhile, the diazirine probe 40 did not establish significant cross-linking to proteins, most likely due to the high reactivity of the transient carbene species, which reacted with and was quenched by water. Further decoration at the fatty acyl chain of probes 13 and 50 with azido groups enabled the conjugation of the fluorescent and biotin tags to the lipid probes via click chemistry, which greatly helped the detection and/or purification of the cross-linked products.⁶⁴ With the aid of these dual functional photoactivatable lipid probes alongside MS analysis, several proteins such as Cox2p and Gut2p were identified to directly interact with PC.⁶⁴ This is in agreement with the findings of crystal structural analysis showing that these membrane protein complexes are tightly bound by PC molecules.^{179–181} New proteins interacting with PC have also been discovered, which are believed to attach peripherally to the mitochondrial inner membrane.⁶⁴ Altogether, the photolabeling approach described here has proven to be efficient at detecting the phospholipid-interacting proteome, thus extending the potential of photoactivatable phospholipids for the study of lipid–protein interactions. It is to mention that recently a bifunctional photoactive fatty acid probe has been developed for profiling and visualization of cellular lipid–protein interactions both in living cells and in a multicellular organism such as the nematode *C. elegans*, offering a new alternative to investigate the lipid–protein interactions.¹⁸²

5.1.2. Cholesterol Probes. Cholesterol represents another indispensable component of eukaryotic cell membranes with the plasma membranes containing up to 50% cholesterol,^{17,183,184} and can form hydrophobic interactions with the fatty acyl chains of the phospholipids in membrane bilayers to reduce the membrane fluidity.^{7,183} Additionally, cholesterol is required in the manufacture of numerous signaling molecules such as steroid hormones as well as in regulating the formation and stability of “lipid rafts” that are believed to function in membrane trafficking and signaling.^{8,185,186} Moreover, cholesterol binds preferentially to various membrane proteins and thus enables them to carry out their biological function. In line with the fundamental roles of cholesterol in mammalian membranes, probes of cholesterol have been invented to investigate cholesterol binding proteins or cholesterol–protein interactions using the photolabeling approach.

Probe 92 (Figure 56) synthesized by Heller et al. in 1979¹⁴³ is such a compound, which bears both photoactivatable and

radioactive moieties with the aim of studying cholesterol–protein interactions. When administered intravenously to rats, the cholesterol probe **92** was found to alter the activities of two membrane-bound enzymes, 3-hydroxy-3-methylglutaryl (HMG)-CoA (NADPH) reductase and acyl-CoA:cholesterol acyltransferase (ACAT).¹⁴³ Further photo-cross-linking experiments of microsomes containing **92** and these two enzymes demonstrated a sharp decline in ACAT activity but unaltered HMG-CoA reductase activity, which provided evidence in support of ACAT being an integral membrane protein. Later research however demonstrated that HMG-CoA reductase does have transmembrane domains and should also be considered as an integral membrane protein.¹⁸⁷ With this in mind, the above results may indicate that the segment of HMG-CoA reductase involved in cross-linkage of probe **92** was not critical for its activity. Further investigation also revealed the interaction site of cholesterol with ACAT and HMG-CoA, hence delivering useful information concerning their topography and the nature of the cholesterol–protein interaction.

Diazirine-based cholesterol probes **86**^{103,188} (Figure 53) and **93**¹⁴⁴ (Figure 56) were also developed to explore cholesterol–protein interactions. Probe **86** was found to fully mimic the behavior of cholesterol with respect to mediating the fusion of Semliki Forest virus and Sindbis virus fusion with target liposomes,¹⁸⁸ while probe **93** could support the growth of ovary cell lines to a level similar to its natural counterpart.¹⁴⁴ Both probes can therefore be suitable for studying cholesterol-binding proteins in membranes.

Myelin is a specialized lipid-rich membrane, which is mainly composed of glycosphingolipids, cholesterol, and a limited spectrum of proteins.^{189,190} Proteolipid protein (PLP), the major myelin protein within the central nervous system (CNS), can be recovered from a low-density CHAPS-insoluble membrane fraction (CIMF).¹⁹¹ Indeed, Simons et al. efficiently cross-linked PLP with the cholesterol probe **86** (Figure 53),¹⁹¹ thus confirming the specific interaction of PLP with cholesterol in myelin and the contribution of cholesterol–protein interactions toward its assembly.

An ingenious experiment was performed by Thiele et al.¹⁰³ to study lipid–protein interactions using concomitantly the cholesterol probe **86** (Figure 53) and the phosphocholine probe **39** (Figure 21)¹⁰³ to label the synaptic-like microvesicles (SLMVs)¹⁹² in the plasma membrane, which contain the cholesterol-binding membrane proteins. The robust photolabeling of membrane proteins with **86** as compared to with **39** disclosed the high affinity and binding capacity of cholesterol for SLMV membrane proteins. Moreover, this finding indicated that the specific interaction between cholesterol and membrane proteins was preferred over that between phospholipid and membrane proteins, suggesting the importance of cholesterol and membrane protein interactions for the formation of SLMVs.

A further application of the probe **86** (Figure 53) in studying lipid–protein interactions was reported by Hamouda et al.¹³¹ They used this probe to inspect the cholesterol-binding domains in the *Torpedo californica* nicotinic acetylcholine receptor (nAChR). Their experiments displayed that the cholesterol probe **86** was able to distribute efficiently in nAChR-enriched membranes and to label two transmembrane segments of nAChR, providing evidence that the cholesterol binding domain of nAChR was situated at the lipid–protein interface.¹³¹ In accordance with this finding, another photolabeling study, this time using **84** (Figure 41), suggested that

the lipid–protein interface of nAChR was the binding domain for cholesterol.¹²⁸

Another cholesterol probe, **93** (Figure 56), was found to be able to mimic natural cholesterol to support the growth of mutant Chinese hamster ovary (CHO) cells.¹⁴⁴ Most importantly, when **93** was delivered into CHO cells as a cyclodextrin complex, it was able to photolabel several discrete polypeptides among which the membrane protein caveolin-1 was recognized. Probe **93** was thereafter regarded as an effective reagent to explore cholesterol–protein interactions in intracellular cholesterol trafficking.

Recently, Hulce et al. developed a chemoproteomic strategy using the clickable, photoreactive sterol probes **98–100** (Figure 62) in combination with quantitative mass spectrometry to

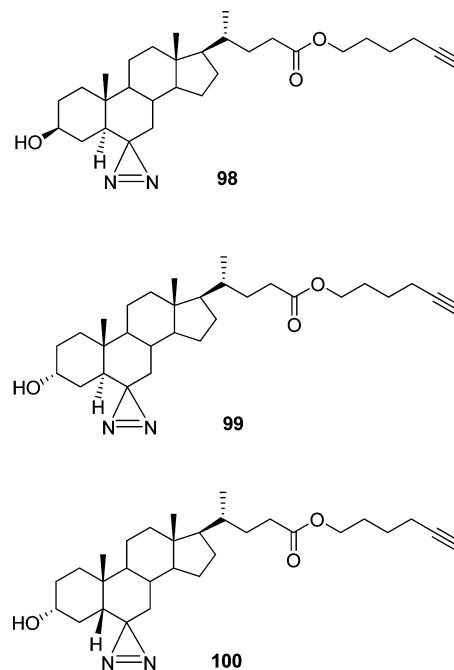


Figure 62. The photoreactive sterol probes **98–100** used for mapping cholesterol-interacting proteins in mammalian cells.¹⁹³

globally map cholesterol–protein interactions directly in living cells.¹⁹³ These probes are, respectively, *trans*-, *epi*-, and *cis*-sterol probes, and all contain a photoactivatable diazirine group at the steroid core and an alkyne group, as a latent affinity handle, incorporated into the cholesterol side chain for conjugation to an azide-reporter tag via click chemistry, hence enabling detection, enrichment, and identification of labeled proteins. With these probes, Hulce et al. identified more than 250 cholesterol-binding proteins in HeLa cells, including those known to biosynthesize, transport, and regulate cholesterol, as well as many others for which no interaction with cholesterol has previously been described.

Considering the higher content of cholesterol in cell membrane and its prominent contribution to maintaining membrane structure and cell function, cholesterol probes have shown their merit as promising tools to study biomembranes and cholesterol-related membrane proteins. Readers are advised to refer to a recent review dedicated to this specific topic for more detailed examples of the use of cholesterol probes in photolabeling studies.¹⁸⁴

5.1.3. Glyco(sphingo)lipid Probes. Glycolipids refer to a class of lipids containing a carbohydrate component and are a

key component of the cell membrane in eukaryotes. The carbohydrate moieties are usually located on the surface of the lipid bilayer where they act as markers for specific cellular recognition, mediate cell signaling, and modulate the activities of proteins.^{194–197} Increasing amounts of evidence gathered over the past years support the metabolism and function of glycolipids being altered upon malignant transformation. These carbohydrate associated lipids appear thus to be involved in multiple diseases and may serve as potential targets for various diseases.^{198,199} Sphingolipids (Figure 2) are an important family of lipids assembled by sphingoid bases and acyl-CoA. They subserve multiple cellular functions such as mediating cell recognition and signaling pathways and have been greatly implicated in the development of multicellular organisms.^{200,201} In higher organisms, most glycolipids are glycosphingolipids.

In 1997, sphingolipid probe **14** (Figure 9) endowed with a *p*-azido-*m*-[¹²⁵I]iodophenyl group⁶⁵ was developed by Zegers et al. with a view to identifying compartment-specific proteins involved in sphingolipid sorting or metabolism.^{200,201} By analyzing the photoproducts issued from **14**, the authors were able to monitor lipid transport in mammalian cells. Moreover, they found that most of the labeled proteins seemed to locate in sphingolipid-rich microdomains at the plasma membrane, thus indicating the potential of applying photoactivatable sphingolipids to further define and identify the role of distinct proteins in sphingolipid-rich microdomains. Recently, to investigate the binding of sphingolipids to transmembrane domains (TMDs) of proteins and how this could affect protein function, Contreras et al. designed an *in vivo* photoaffinity labeling study to evaluate the specific interaction of one sphingomyelin, SM 18, with the TMD of the protein p24.²⁰² They labeled Chinese hamster ovary (CHO) cells with a photoactivatable sphingosine **101**²⁰³ (Figure 63) and analyzed the labeled proteins using Western blotting as well as digital autoradiography, and revealed a strong sphingolipid labeling of p24.

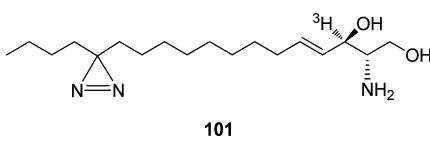


Figure 63. Photoactivatable sphingosine **101** for the study of lipid–protein interaction.^{202,203}

Glucosylceramides (GlcCer) (Figure 64) are a particular group of glycosphingolipids bearing a single glucose attached to the polar head, based on which Zegers et al. developed probe **102** (Figure 64).⁶⁵ In parallel, Pacuszka et al. were interested in photoactivatable glycosphingolipids aimed at defining glycolipid function.¹³⁰ They prepared the corresponding photoactive probe **85** (Figure 44) to label human erythrocyte membranes. Their findings showed that about 50% of the probes could firmly bind to erythrocytes with a distribution ratio between membrane proteins and lipids of about 1:2.3. In addition, several membrane components were labeled efficiently.

Prioni et al. developed the photoactivatable GM1 ganglioside analogue **103** (Figure 64) to exploit the interactions between gangliosides and proteins at the exoplasmic surface of the sphingolipid-enriched membrane domains.²⁰⁴ After the photo-labeling by **103**, several proteins cross-linked to **103** were found to bear an exoplasmic domain, among which a GPI-anchored neural cell adhesion protein was identified.²⁰⁴ These data

therefore suggested the existence of interactions between the exoplasmic domains of the protein and the ganglioside sialo-oligosaccharide chain.

Altogether, the above-mentioned examples firmly illustrate the considerable contribution made by photoactivatable lipid probes to the study of lipid–protein interactions, the data from which have expanded our knowledge of biomembranes.

5.2. Membrane Proteins

As one of the most important components in biomembranes, membrane proteins are extremely valuable subjects to study not only in terms of their biological significance but also for their structural diversity.¹⁹ According to their location in the lipid bilayer, membrane proteins are sorted into integral and peripheral proteins (Figure 1). In addition, membrane proteins can be classified according to their function in different biological processes, for instance, as receptors, transporters, ion channels, etc. As lipids constitute the essential components of biomembranes and their presence in membranes is crucial for membrane proteins to maintain their stable conformation and exercise the corresponding function, competent lipid probes are important for photolabeling investigations on biomembranes. We feature below the applications of various lipid probes in photolabeling studies on such membrane proteins arranged according to their function.

5.2.1. Receptors. Receptors are a class of proteins that can receive and/or respond to signals from either the internal or the external environment. A large number of receptors are membrane proteins, and they are either embedded in or associated with biomembranes.²⁰⁵ These membrane protein receptors facilitate the binding of the signal molecules, thereby promoting their molecular recognition and signal transduction, and as such play a critical role in inter- and intracellular communication and organism metabolism, etc. We will focus in particular on certain examples in which the photolabeling approach was used to study glycophorins and nicotinic acetylcholine receptors, two membrane proteins that have been the focus of a variety of investigations due to their easy access and prominent biological functions.

5.2.1.1. Glycophorins. Glycophorins are a group of transmembrane proteins found in red blood cells (RBCs) and function as receptors for the binding of various ligands such as blood group antigens to execute transmembrane communication.^{206–209} Among the different glycophorins, glycophorin A (Figure 65) is the most abundant,²¹⁰ and its amino acid sequence was first determined in the 1970s.^{211,212} Glycophorin A is decorated with oligosaccharides bearing sialic acids, which contribute to the negative surface charge of the cell membrane that prevents red blood cells from aggregating during their circulation.²⁰⁶ Historically, glycophorin A has served as a reference protein in topological studies of transmembrane proteins due to its relative abundance in red blood cells and easy enrichment and purification.

Khorana and co-workers developed the phospholipid probes **36–38** (Figure 21) with the aim of characterizing the membrane-embedded region of glycophorin A in the lipid bilayer.¹⁰² They reconstituted glycophorin A into vesicles composed of dimyristoylphosphatidylcholine (DMPC) and one of these photoactivatable probes. Photoirradiation led to the covalent cross-linking with glycophorin A. Enzymatic degradation of the labeled protein into small peptides together with subsequent amino acid sequence analysis led to the following findings: the hydrophobic chain-modified probe **36** labeled

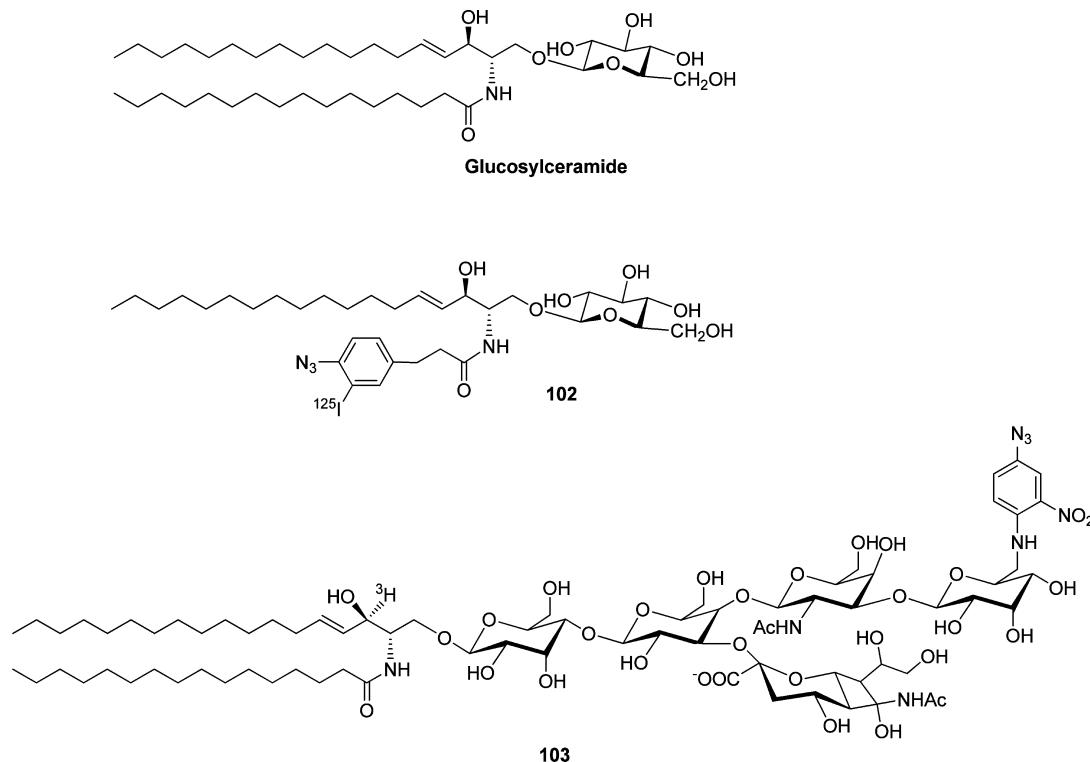


Figure 64. Glucosylceramide (GlcCer), and the photoactivatable probes **102** and **103**.^{65,130,204}

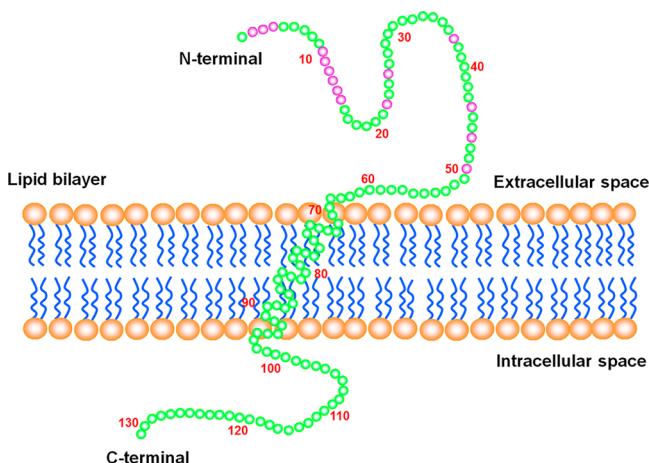


Figure 65. Schematic representation of glycophorin A. Glycophorin A is a membrane protein with 131 amino acid residues. The amino acid residues are denoted as green and purple dots, with the purple dots representing amino acid residues with O-linked oligosaccharides.

Glu70, which was located within the lipid bilayer, whereas the two polar head-modified probes 37 and 38 produced cross-linking on residues 62–81 and 82–96, respectively, both belonging to the lipid–water region of the membrane part of glycophorin A.

Brunner et al. also photolabeled glycophorin A within the human erythrocyte membrane using the ³H-labeled lipid probe 42 (Figure 21).¹⁰⁴ They inserted 42 into the erythrocyte membrane, which photo-cross-linked various membrane components including glycophorin A. Their results showed that 42 mainly labeled the fragments 9–81 and 82–131 of glycophorin A, with labeling sites being located in the transmembrane domain.

Moreover, Ourisson and co-workers used the bola-lipid **59** (Figure 29)^{117,213} to inspect the topography of the transmembrane domain of glycophorin A. Their findings showed that **59** was completely incorporated into DMPC vesicles accompanied with complete miscibility in fluid phase. In addition, the benzophenone chromophore appeared to be localized inside the lipid bilayer and could therefore efficiently and regioselectively photolabel near the middle of the membrane. To further expedite the cross-linking product analysis, they used probes **90** and **91**, which contain ³H and ¹²⁵I as radioactive tracers, respectively (Figure 55).¹³² The reconstitution of glycophorin A in liposomes composed of these probes and other membrane lipid components was confirmed by electron microscopy.¹³² Further proteinase-K digestion of the reconstituted liposomes suggested that the orientation of glycophorin A in the reconstituted proteoliposomes was virtually identical to that observed in natural erythrocyte membranes.¹³² Photoirradiation of the reconstituted proteoliposomes together with tryptic digestion led to the identification of the covalent labeling of the amino acid residues Val80 and Met81.¹³² This finding not only confirmed the results previously obtained by Khorana and Brunner, but also indicated that both Val80 and Met81 are located near the center of the transmembrane domain of glycophorin A, demonstrating the power of transmembrane bola-lipid probes in region-specific labeling of biomembranes.

5.2.1.2. Nicotinic Acetylcholine Receptors (*nAChRs*). Nicotinic acetylcholine receptors (*nAChRs*) are pentameric integral membrane proteins with subunits of diverse subtypes (Figure 66),^{214,215} and have been extensively studied using the photolabeling approach. In fact, they represent the best-studied superfamily of Cys-loop membrane proteins, which function as ionotropic receptors to regulate a variety of physiological processes, such as neurotransmission, ion transportation,

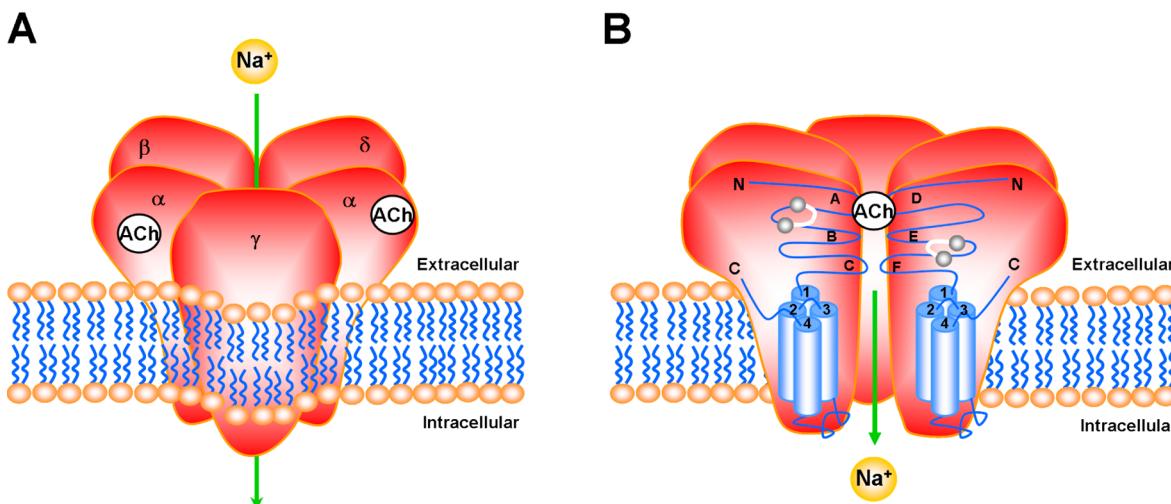


Figure 66. Structure of the nicotinic acetylcholine receptor (nAChR). (A) The five subunits that make up the nAChR receptor are arranged around a central channel, which is permeable to cations such as Na^+ . (B) Schematic representation of the heteromeric nAChR receptor. The polypeptide layout of two subunits is shown with the Cys-loop (presented by two gray dots with a white bridge), which defines the α -subunit and four transmembrane domains (TM1–4). The six binding loops (A–F) contribute toward the binding of acetylcholine (ACh).^{214,215} Adapted from ref 215 with kind permission of Springer Science+Business Media. Copyright 2010 Springer Science+Business Media.

etc.^{214,216} As nAChRs exist widely in the nervous system and mediate communications in complex nervous activity, they represent important targets for neurodegenerative diseases, and their ligands are potential candidates to treat various central nervous system diseases.²¹⁷ Concerning the significant importance of nAChRs in structural biology and drug development, a wide diversity of photoactivatable lipids has been applied to study these receptors.

The most common lipid analogues used to study nAChRs are cholesterol probes because cholesterol is required for the function and the stabilization of nAChRs.^{218,219} As mentioned in the above section on lipid–protein interactions, Hamouda et al. used the cholesterol probe **86** (Figure 53) to identify the cholesterol-binding domains in the *Torpedo californica* nAChR.¹³¹ In fact, prior to this work, Corbin et al. had already used a cholesterol probe, **84** (Figure 41), to label nAChR in receptor-rich native membrane and affinity-purified nAChR in reconstituted vesicles.¹²⁸ In both cases, all nAChR subunits incorporated with probe **84**, and the presence of the agonist carbamoylcholine did not affect the labeling. This suggests that the agonist-induced structural transition does not significantly change the surface area of nAChR exposed to the cholesterol. Further investigation on the V8 protease digested subunit fragments demonstrated that the labeling of **84** was localized on peptides containing almost exclusively the membrane-spanning segments: TM1 and TM4 of the α -subunit as well as TM4 of the γ -subunit. As these segments form the lipid–protein interface of the nAChRs,²²⁰ these results indicated that the binding domain for cholesterol is located at the lipid–protein interface of the nAChRs.

Fernandez et al. used probe **104** (Figure 67),²²¹ which carries the photolabeling group at the hydrophobic tail of cholesterol, to study nAChRs in reconstituted vesicles. Again, all subunits of the nAChRs were labeled by **104**. Moreover, they found that the presence of neutral phospholipids could reduce the extent of photolabeling of **104** in nAChR. This indicated that the potential interaction sites for cholesterol **104** in nAChRs were probably occupied by the neutral phospholipids. In the presence of cholinergic agonists such as carbamoylcholine,

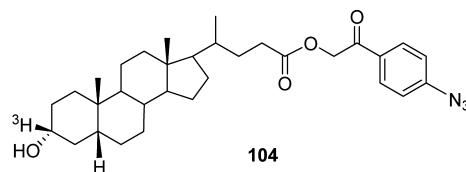


Figure 67. Cholesterol probe **104** developed by Fernandez et al.²²¹

the photolabeling experiment provided evidence that probe **104** was capable of sensing nAChR desensitization induced by exposure to the agonist.

In addition to the photoactivatable cholesterol, the phosphatidylserine probe **11** (Figure 9) with a radioiodinated aryl azido group at the polar head was also applied to label nAChRs.⁶³ Although probe **11** tended to label all of the subunits of the nAChRs, the data showed a greater incorporation on the α -subunit as compared to the others, which was later found to be localized to the V8 cleavage fragments extending from both the Asn339-containing hydrophobic region TM4 and the Ser173-containing regions TM1, TM2, and TM3. Soon after, the same authors examined the binding domains of **11** in nAChRs,¹⁶⁷ which were identified to contain the flanking regions of TM4 as well as TM3. Because the photoactivatable group of **11** is located at the phosphatidylserine head, the labeled regions were believed to preferentially interact with the lipid/water interface. This therefore suggested that the hydrophobic faces of TM3 and TM4 within the transmembrane helices of nAChRs could constitute the boundary of the receptor complex in contact with the lipid bilayer.

Apart from glycophorins and acetylcholine receptors, other membrane receptors have also been investigated by photoaffinity labeling, such as the cannabinoid receptor 1 (CB1),²²² sphingosine 1-phosphate type-1 receptor (S1P1),²²³ platelet-activating factor receptor (PAFR),²²⁴ and endotoxin receptor,²²⁵ etc. However, none of these studies involved lipid probes, and we shall therefore not discuss them here.

5.2.2. Transporters. Biomembranes not only provide a platform for the communication and transport between cell

compartments but also form a hub for many important biological events such as the production and transportation of substances essential for cell function, which involves the active participation or imperative action of enzymes.²²⁶ Membrane proteins also act as enzymes to catalyze various biological reactions and regulate diverse metabolic pathways. Studies using the photolabeling approach with lipid probes on these membrane-associated enzymes, which mainly act as transporters in biomembranes, have permitted considerable advances in understanding the functional role of biomembranes.

5.2.2.1. Plasma Membrane Calcium Pump. The plasma membrane calcium pump is an integral membrane protein possessing calcium-dependent ATPase activity (PMCA) (Figure 68).²²⁷ It functions vitally to regulate the basal Ca^{2+}

Extracellular space

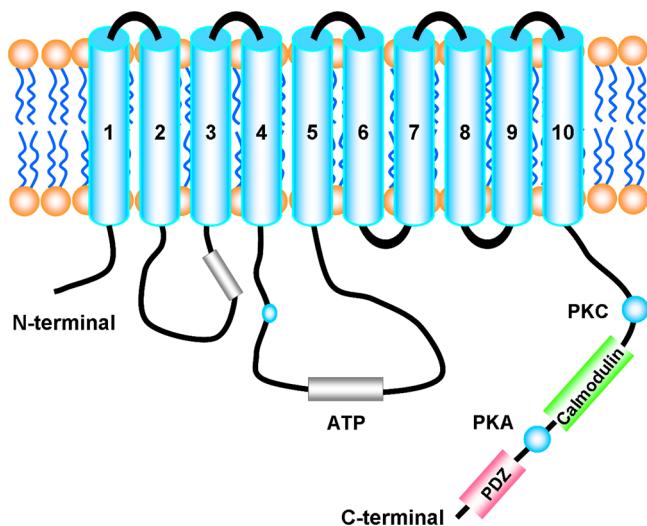


Figure 68. Schematic presentation of the membrane topology of the PMCA pump. The 10 transmembrane domains (TM) are symbolized by cylinders numbered 1–10.²²⁷ Adapted with permission from ref 227. Copyright 2008 Elsevier.

in the cytoplasm of eukaryotic cells to control global Ca^{2+} homeostasis and intracellular Ca^{2+} signaling.^{3,227–229} Because Ca^{2+} plays an essential role in various cellular processes²³⁰ including cell survival, apoptosis, angiogenesis, as well as gene transcription relevant to tumorigenesis, PMCA as the regulator of Ca^{2+} efflux is currently regarded as a potential therapeutic target for cancer treatments.²³¹ Investigating the structure of PMCA together with its interaction with the Ca^{2+} binding protein calmodulin and the surrounding phospholipids will be instrumental to the successful design of pharmacological agents that target PMCA.

PMCA comprises very large transmembrane domains TM1–TM10 as shown in Figure 68. To examine the membrane-associated regions of human erythrocyte PMCA, Delfino et al. performed a photolabeling study using the ^3H -radiolabeled bola-lipid 89 (Figure 55).^{50,232} Their results showed that the labeled polypeptide fragments were clustered around three distinct regions, which consisted of TM1–TM2, TM3–TM4, and TM5–TM10, respectively. These data proved the existence of the hydrophobic clusters, which supported the established model for PMCA topography.²³³ Similarly, Giraldo et al. focused on using the probe 88 (Figure 54) to verify the

distribution of various lipids among different membrane-associated regions of PMCA.¹⁶⁵ The results of lipid–protein stoichiometry obtained from this work provided the number of lipid-associated sites in PMCA. This example has already been cited in the above section of lipid–protein interactions.

Recently, Mangialavori et al. further exploited 88 (Figure 54) to assess the exposure of PMCA to the surrounding phospholipids.²³⁴ They found that, in the presence of Ca^{2+} , photolabeling of PMCA by 88 was highly decreased by calmodulin and phosphatidic acid, two important modulators of ATPase activity within PMCA. This finding indicates that conformational changes induced by the binding of calmodulin or phosphatidic acid extended to the adjacent transmembrane domains. Their work also disclosed the existence of differences between calmodulin and phosphatidic acid-induced PMCA conformations and identified the involvement of the transmembrane regions in these differences.

5.2.2.2. Sec Translocase. In addition to the enzymes in eukaryotic cell membranes, those in bacteria have also been studied using the photolabeling approach. For example, in *Escherichia coli*, the translocation of proteins across the cytoplasmic membrane is catalyzed by Sec translocase, a multimeric membrane protein complex (Figure 69).^{235–237} The

Cytoplasm

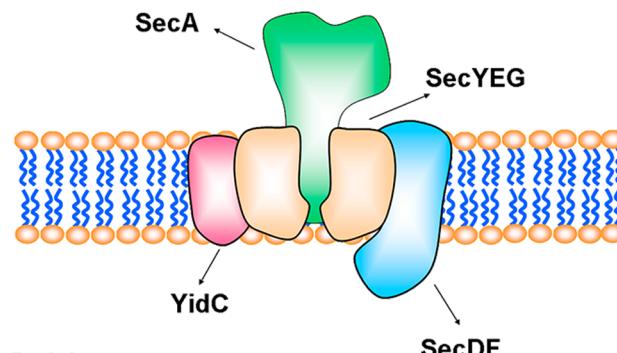


Figure 69. Schematic representation of the bacterial Sec translocase. This cytoplasmic membrane protein complex comprises a peripheral motor domain SecA (green), the protein-conducting channel SecYEG (orange), and the accessory proteins SecDF (blue) and YidC (pink).²³⁸

Sec translocase is composed of a highly conserved protein-conducting channel, SecYEG, an ATP-dependent motor protein SecA, and the accessory proteins SecDF and YidC.^{237,238} Although SecA has a clear functional role in protein secretion and in coupling ATP to protein translocation, doubt remains over whether SecA inserts in the membrane via a lipid environment or through the SecYEG-formed pore. van Voorst et al. therefore used the lipid probe 88 (Figure 54) to investigate the lipid accessibility of SecA bound at the translocase.²³⁹ Their data suggested that the SecYEG-bound SecA was largely shielded from the phospholipid acyl chains and that two distinct pools of membrane-bound SecA existed with different degrees of lipid association. These results indicated the involvement of phospholipids in translocation via their action on the lipid-bound fraction of SecA and shed some light on the structure of the secretion machinery in prokaryotic cells.

5.2.3. Ion Channels. Ion channels are a family of transmembrane proteins, which render selective the transportation of ions across cell membranes and regulate the communication between a cell and its environment.^{240–242} They are actively involved in many critical biological processes, such as sensing and signaling pathways. Many ion channels are also drug targets for diseases including cancers and neurodegenerative diseases.^{25,243} Studies on ion channels are therefore of high biological and clinical importance. Here, we mainly focused our review on the photolabeling study of ion channels including gramicidin A, porins, and voltage-dependent anion channels. It is to note that the nAChRs described above also belong to the family of ligand-gated ion channels based on their structural and functional properties in addition to their function as neurotransmitter receptors.²¹⁹ They are not included in this section to avoid redundancy.

5.2.3.1. Gramicidin A. Gramicidin A is the simplest ion channel formed by a peptide composed of 15 amino acids (Figure 70A). This peptide is able to assemble, via head-to-head

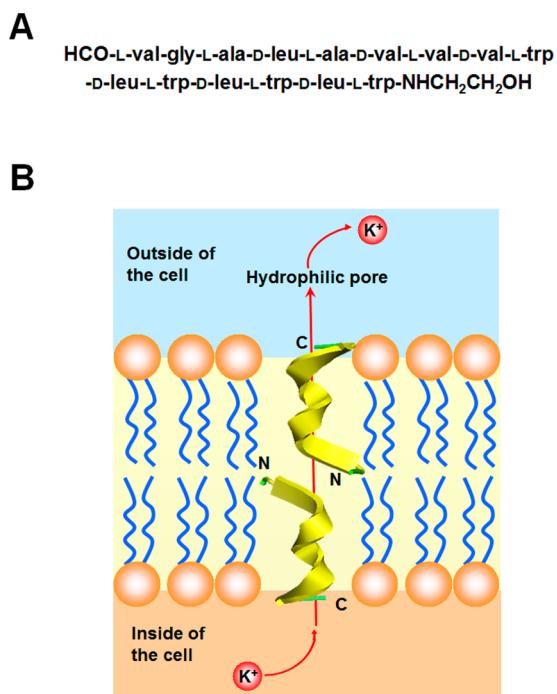


Figure 70. (A) The amino acid sequence of gramicidin A.²⁴⁴ (B) A schematic representation of the channel formation of gramicidin A in a membrane bilayer. Adapted with permission from ref 246. Copyright 2007 Elsevier.

dimerization, into a transmembrane channel, allowing ions, such as H⁺, Na⁺, K⁺, to pass through the membrane (Figure 70B).^{244–246} As one of the most recognized ion channels, gramicidin A serves as the classic model to study the organization, dynamics, and function of membrane-spanning channels.²⁴⁶

Brunner et al. studied gramicidin A using the three different photoactivatable phospholipid probes 7, 8 (Figure 9), and 41 (Figure 21), which contain either a *p*-azidophenyl or a *p*-(3-trifluoromethyl)diazirino)phenyl group in the hydrophobic aliphatic chain.⁶⁰ Probe 41 displayed superior tendency over 7 and 8 for cross-linking the multilamellar liposomes composed of phosphatidylcholine, while the nitrene-generated probe 8 proved to be more efficient in photolabeling gramicidin A. This

is because the phenylnitrene is a poor reagent for C–H bond insertion into the lipids and hence prefers cross-linking with peptides; whereas the more reactive carbene generated from 41 favors both C–H insertion and peptide labeling, with the former reaction consuming significantly more of the carbene, thereby leading to lower labeling yields of the peptide. Further amino acid analysis and Edman degradation sequencing revealed the indole rings of the tryptophan residues in gramicidin A as the most likely sites of labeling by 8, whereas no detectable C–H insertion in lipids could be found with 8.

An additional structural study on gramicidin A was achieved with the bola-lipid 46 (Figure 23) bearing the carbene precursor, (trifluoromethyl)phenyldiazirine.⁵⁰ This membrane-spanning probe developed by Delfino et al. was expected to label the transmembrane region in gramicidin A, especially the N-terminal region of the peptide dimer located close to the center of the membrane. However, as the reaction with the indole ring of tryptophan is the dominant reaction of carbenes, 46 preferentially labeled tryptophan residues located in the C-terminal region of gramicidin A.⁵⁰ Delfino et al. went on to prepare a peptide analogue of gramicidin A in which the Val residue at the N-terminal was replaced by Trp. This substitution permitted the doubling of photoadduct yield.⁵⁰ A much reduced rate increment was observed in the event of a competition with the reaction at the Trp residues located in the C-terminal half.⁵⁰ This result therefore illuminated the geometrical regioselectivity of bola-lipid and the chemical reactivity of diazirine within the probe 46 in photolabeling gramicidin A.

5.2.3.2. Porins. Porins are the principal channels in gram-negative bacteria.^{247,248} They are a class of integral membrane proteins found in the outer membrane of the bacteria^{249,250} and have β-barrel structures with a hydrophobic outer surface contacting the surrounding lipids and a hydrophilic inner surface forming the pore, providing the channel for the passage of different solutes across the membrane (Figure 71).^{247,248} Page et al. explored the topography of three porins from *Escherichia coli* by photoaffinity labeling using phospholipid probes 6 (Figure 8) and 49 (Figure 27).¹¹⁴ Probe 49 displayed preferential labeling of porins at the regions in contact with the hydrophobic core of the membrane. Because porins are

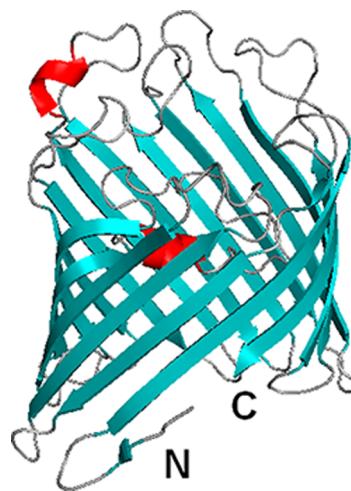


Figure 71. General fold of a porin monomer (OmpF porin from *E. coli*). The large hollow β-barrel structure is formed by antiparallel arrangement of 16 β-strands.^{251,252}

membrane-embedded proteins, it is easily understandable that **49** exhibited superior labeling efficiency as compared to the head-modified probe **6**.

5.2.3.3. Voltage-Dependent Anion Channels. Voltage-dependent anion channels (VDACs) are key players in a variety of physiological processes, including cell metabolism, energy conversion, and cell apoptosis, etc.²⁵³ These highly voltage-dependent channel proteins are located in the mitochondrial outer membrane and are structurally similar to bacterial porins, hence being also named mitochondrial porins (Figure 72). These channels function to regulate the exchange

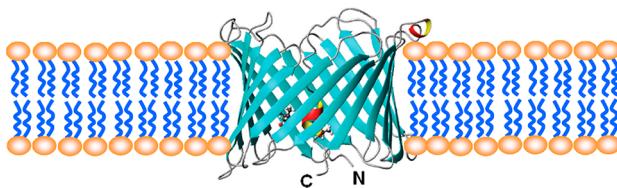


Figure 72. Voltage-dependent anion channel 1 (VDAC-1), or mitochondrial porin, is located on the outer mitochondrial membrane.²⁵⁷ Adapted with permission from ref 257. Copyright 2008 The American Association for the Advancement of Science (AAAS).

of ions and small molecules into mitochondria, and thereby allow the communication between mitochondria and other cellular components.^{253,254} In 2003, Darbandi-Tonkabon et al. applied the steroid probe **105** (Figure 73) to inspect the

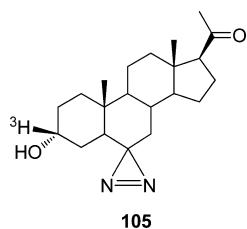


Figure 73. Sterol probe **105** used to study neuroactive steroid binding sites.²⁵⁵

neuroactive steroid binding sites in rat brain membranes.^{255,256} Their results showed that **105** could produce photolabeling on two proteins with molecular weights of 35 and 60 kDa, respectively, in a time- and dose-dependent manner. 2D-PAGE followed by MS analysis indicated that the 35-kDa protein was VDAC-1, which was further confirmed by immunoprecipitation assay with a monoclonal antibody. Moreover, VDAC-1 could be coimmunoprecipitated with GABA_A, an important receptor in the central nervous system, indicating a physical association between VDAC-1 and GABA_A, which might mediate neuroactive steroid effects on the GABA_A.

In this section, we have reviewed examples of photolabeling studies on membrane proteins using various lipid probes. The above cited examples have principally focused on proteins that are relatively stable and abundant, allowing reasonably easy and convenient separation and purification, and hence successful photoaffinity labeling. It is also important to note that several factors contribute to the labeling outcomes of these membrane proteins: (1) On the basis of the natural characters of lipids, different types of probes (such as phospholipid or cholesterol) have been chosen to label different proteins. (2) Lipid probes with photolabeling groups either at the hydrophobic region or

at the hydrophilic region enable the labeling on water–lipid interface or transmembrane part of membrane proteins. (3) The properties of the reactive species (nitrenes, carbenes, or radicals) generated by the photoactive groups may influence the labeling tendency of the probes to their surrounding lipids or membrane proteins. Therefore, the choice of lipid, photolabeling group, as well as the location of the groups within the lipid structure should be carefully considered for each particular study. Moreover, the above examples demonstrate that combination labeling with different photoactivatable lipid probes can deliver more insightful information on biomembranes. Although numerous other approaches have also been used to assess these membrane proteins, photoaffinity labeling with lipid probes is considered to complement the existing techniques and afford a direct route to studying membrane proteins within biomembranes.

5.3. Membrane Fusion

In the above sections, we have reviewed the successful application of lipid probes in the study of lipid–protein interactions and membrane proteins. Another important purpose for these probes is to analyze the proteins involved in certain biomembrane dynamic processes such as membrane fusion²⁵⁸ during viral infection, in particular, influenza viral membrane fusion, which has been extensively examined using the photolabeling approach.

Virus infection proceeds via fusion of the viral and the host cell membrane, a process catalyzed by viral fusion proteins, followed by the injection of the viral genome into the host cell.²⁵⁹ In this process, influenza virus hemagglutinin (IVH) behaves as a viral fusion protein to facilitate the membrane fusion step for influenza virus infection. IVH is a glycoprotein in the influenza virus membrane and is characterized as a trimeric protein with three identical subunits.^{259,260} Each subunit of the trimeric hemagglutinin (HA) contains two polypeptides called HA1 and HA2 (Figure 74).²⁶¹ A hydrophobic membrane-spanning segment at the C-terminal of HA2 helps anchor the protein complex to the viral membrane.

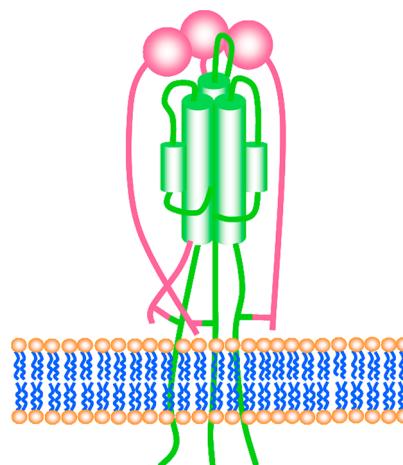


Figure 74. Schematic presentation of the trimeric organization of viral hemagglutinin (HA) in the native conformation. The globular headgroups of HA1 subunits (pink) sit on top of the long helix of HA2 subunits (green). The HA1 subunit is linked to the HA2 subunit by a disulfide bond at the bottom of the subunit close to the viral membrane. The HA trimer can facilitate the attachment of virus to host via HA1 subunits.^{261,262} Adapted with Dr. Peter S. Kim's permission from ref 262.

Viral membrane fusion is triggered by low endosomal pH. With the aim of exploring the molecular basis for such low-pH-mediated membrane fusion, Harter et al. photolabeled BHA, the water-solubilized ectodomain of HA prepared by bromelain digestion, with probe 43 (Figure 21).¹⁰⁵ They demonstrated that the labeling of BHA was strictly pH-dependent and selectively incorporated into the HA2 polypeptide, named as BHA2. This finding indicated that the interaction of BHA with membranes was mediated through BHA2, probably via its hydrophobic N-terminal segment. Further work by Tsurudome et al. demonstrated that the labeling of the BHA2 polypeptide chain correlated remarkably with the temperature dependence of the fusion activity of whole viruses.²⁶³ A temperature-induced structural change appears to be critical for both the interaction of BHA with membranes and the expression of fusion activity of intact viruses.

To further investigate the interaction between the influenza virus and target membrane lipids, a photolabeling study was performed using the bola-lipid probe 89 (Figure 55).⁵⁰ 89 was loaded into lipid vesicles, which served as a model of the target host for fusion with the virus. Experimental results revealed that the fusion between virus and vesicles occurred readily and the subsequent photolysis led to the labeling of viral HA. Moreover, the labeling was associated with the membrane-spanning polypeptide domain of HA (HA2) only and not with extra-membrane portions. Further study using probe 89 demonstrated that the HA2 subunit likely inserts into the target membrane prior to the fusion step and indicated that the direct interaction of the HA2 with the lipids of the target membrane would trigger the fusion.²⁶⁴ Photolabeling studies on the mode of action of IVH have indisputably enhanced our understanding of influenza virus infection.

Overall, obtaining structural and functional information from biomembranes during various biological processes is undoubtedly critical to achieving a complete understanding of the global role of biomembranes. As we have endeavored to highlight in this Review, photolabeling study using photoactivatable lipid probes are proving valuable in this regard.

6. CONCLUSIONS AND PROSPECTS

Since the first photoactivatable lipid was invented in the 1970s,²⁹ various lipid probes have been developed, which have greatly accelerated the structural and functional study of biomembranes via a photolabeling approach. To achieve a successful photolabeling study, the design and synthesis of stable yet highly efficient photoactivatable lipid probes remains the primary concern and is being actively pursued. Nitrenes, carbenes, carbocations, and radicals are the most frequently reported reactive intermediates in photoaffinity labeling, and endow the lipid probes with distinct photochemical properties. Coupling the respective chromophores to different locations on the lipid skeleton provides a valuable tool for photoaffinity labeling studies of the specific regions of biomembranes. In addition to selecting the suitable photolabeling groups to construct different lipid probes, the characters and contents of lipids in biomembrane should be taken into account. As PC is often more abundant than PE or PS in biomembranes, more PC-based lipid probes have been developed for photolabeling studies of the biomembrane. Cholesterol represents another important component of eukaryotic cell membranes and contributes crucially to the formation of lipid rafts. Therefore, the photoactivatable cholesterol probes are considered as efficient tools to label membrane proteins that interact with

cholesterol, as well as specific regions in lipid rafts. It is worth noting that the additional decoration of lipids with different tags, such as a fluorescent moiety or biotin, can facilitate the detection and purification of the photo-cross-linked products. The photolabeling approach combined with analytical techniques, such as electrophoresis, HPLC, MS, etc., has allowed the effective identification of the labeled proteins or lipids, offering valuable information to enhance our understanding of biomembranes

Despite much progress in the development of photolabeling lipids, many limitations remain. Often photolabeling studies using a single lipid probe may not be able to offer detailed and insightful information about biomembranes. To overcome this limitation, the use of several probes of different lipid type with different photolabeling groups located at different regions of the lipid may be necessary to deliver a more comprehensive view. Moreover, many biological events on biomembranes are not static, but rather dynamic. A method of choice to tackle this problem can be achieved using time-resolved photolabeling study provided it is allowed by the dynamic character of the biological phenomena under study.

Finally, one outstanding issue in the study of biomembranes is how to perform the photolabeling approach in real biological systems as opposed to in artificial reconstituted membranes. Our current understanding of biomembranes is mostly based on studies performed on *in vitro* reconstituted membrane models. Strategies aimed at establishing a native biomembrane environment for photolabeling are therefore paramount to obtaining more reliable and credible structural data on biomembranes. Although various technological innovations such as high-resolution NMR,^{15,265–267} X-ray crystallography,^{28,268} electron crystallography,^{269–271} mass spectrometry,^{168,169,272} and electron spin resonance (ESR)^{273,274} have considerably accelerated the pace of research on membrane protein structures and other biomembrane related events, they can seldom be used to unravel the structures of membrane proteins in their native environment. Much effort has thus been devoted to the development of novel techniques either able to overcome the limitations encountered in the structural study of biomembranes or to complement the currently predominant techniques. These have included protein engineering,¹⁴ micro-focus X-ray diffraction,¹⁴ and femtosecond nanocrystallography.²⁷⁵ We believe that further development of photoactivatable lipid probes and the successful harnessing of multidisciplinary techniques designed to complement the photolabeling will continue to impel the investigation of biomembranes and the related biologically important events.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ling.peng@univ-amu.fr.

Notes

The authors declare no competing financial interest.

Biographies



Dr. Yi Xia was trained as a synthetic chemist at Wuhan University, China. Her Ph.D. thesis dealt with the synthesis and characterization of photoactivatable phospholipid probes for photolabeling study of biomembranes. After receiving her Ph.D. in 2007, she was honored with a short-term visiting scholarship from the French embassy in China, before obtaining a postdoctoral fellowship from the Fondation pour la Recherche Médicale to undertake her postdoctoral research with Dr. Ling Peng at the Interdisciplinary Center of Nanoscience in Marseilles (CNRS UMR 7325) at Aix-Marseilles University, France. Her research interests are focused on using lipidic probes to investigate biological events and developing small molecular drug candidates for anticancer study.



Dr. Ling Peng is a research director within the French scientific research institute CNRS. She received her undergraduate degree in chemistry at Nanjing University, China, before joining the group of Professor Albert Eschenmoser at the Swiss Federal Institute of Polytechnic in Zurich for her Ph.D. program. After completing her Ph.D. thesis, she spent three years as a postdoctoral researcher within the group of Prof. Maurice Goeldner at the Louis Pasteur University of Strasbourg, France, where she developed various photosensitive probes to investigate the functions of cholinesterases and biomembranes. In 1997, she was recruited as a research scientist by the CNRS to undertake research at the interface of chemistry and biology. She is currently a group leader at the Interdisciplinary Center of Nanoscience in Marseilles, France. Her current research mainly centers around developing chemical tools for exploring biological events and drug discovery as well as dendrimeric nanovector-based drug delivery.

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