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Review

Tandem photoaffinity labeling–bioorthogonal conjugation in medicinal chemistry

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ABSTRACT

Photoaffinity labeling has a longstanding history as a powerful biochemical technique. However, photoaffinity labeling has significantly evolved over the past decade principally due to its coupling with bioorthogonal/click chemistry reactions. This review aims to highlight tandem photoaffinity labeling–bioorthogonal conjugation as a chemical approach in medicinal chemistry and chemical biology. In particular, recent examples of using this strategy for affinity-based protein profiling (AfBPP), drug target identification, binding ensemble profiling, studying endogenous biological molecules, and imaging applications will be presented. Additionally, recent advances in the development of 'all-in-one' compact moieties possessing a photoreactive group and clickable handle will be discussed.

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1. Introduction

Since their inception more than 45 years ago, photoaffinity ligands continue to evolve as valuable compounds in medicinal chemistry and chemical biology. Given the longstanding history of photoaffinity labeling as a biochemical technique, a number of prominent reviews have been dedicated to this topic,[1–3] particularly from a drug discovery and drug development perspective.[4,5] Traditionally, photoaffinity ligands permit researchers to interrogate the structure and function of biological macromolecules (e.g., receptors, enzymes), identify the target(s) of biologically active compounds (i.e., macrolevel analysis), determine the selectivity and affinity of ligand–target complexes, and three-dimensionally map ligand-binding sites within biological targets (i.e., microlevel analysis). However, photoaffinity probes have undergone significant advancement within the past decade, principally due to the advent of click chemistry and bioorthogonal reactions.[6–8] The primary objective of this review is to introduce synthetic organic medicinal chemists and chemical biologists to the most recent accomplishments involving tandem photoaffinity labeling–bioorthogonal conjugation as a chemical strategy.

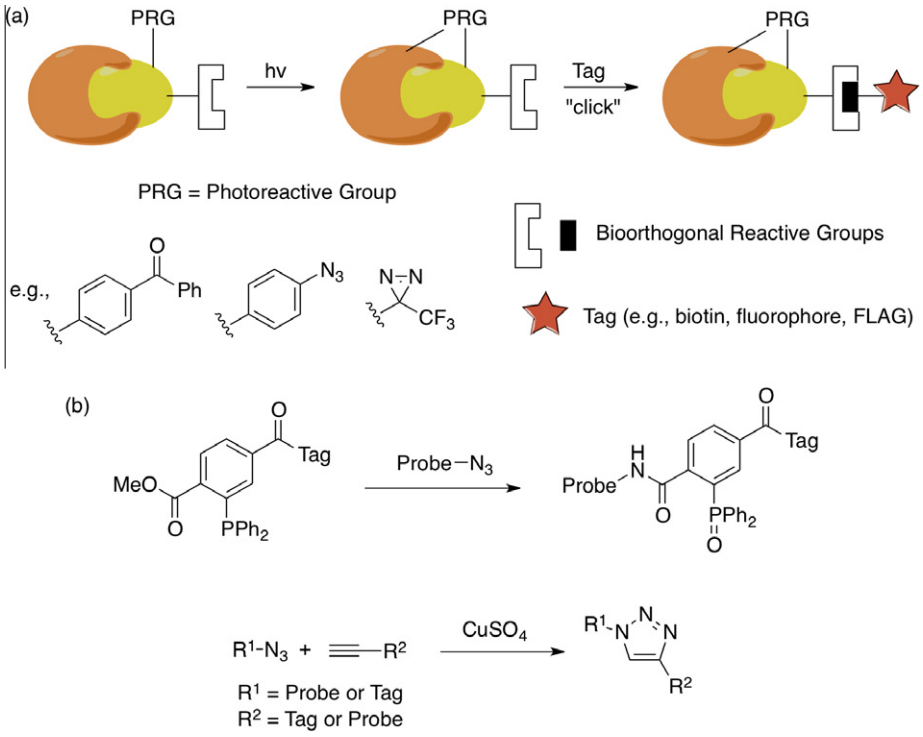
1.1. Photoaffinity labeling

In photoaffinity labeling, a compound is derivatized with a photoreactive group (PRG) that can be irradiated with UV light (hv) upon reversible or irreversible complexation with a biological target (Step 1 in Scheme 1a). Photoirradiation facilitates the formation of a highly reactive intermediate, typically a nitrene, carbene, or diradical, which is capable of forming a covalent bond between the biological target and the ligand/photoprobe. In particular, aromatic azides, aliphatic[9] and aromatic diazirines,[10–12] and benzophenones have been widely used as photoreactive groups in photoaffinity labeling. In short, each of these groups possesses certain advantages and disadvantages. For example, a major drawback of aryl azides is that their maximum absorption wavelength is <300 nm, which could result in substantial damage to the biological system upon photoirradiation. However, aryl azides are relatively small, and can be easily prepared and incorporated into a ligand of choice. In contrast, diazirines absorb most efficiently at 350–380 nm, therefore no significant damage to the biological system is expected during photoirradiation. Additionally, diazirines are stable, unlike aryl azides, to a wide range of chemical conditions including strongly basic, strongly acidic, oxidizing, and reducing agents. However, a potentially inherent disadvantage of diazirines is that their syntheses can be somewhat lengthy and complicated. Finally, benzophenones are typically excited at 350–360 nm (i.e., similar to diazirines), with a number of commonly used building blocks commercially available. However, benzophenones are notoriously bulky functional groups, which could negatively influence proper interactions between the photoprobe and its biological target(s).

It is beyond the scope of this report to further delineate the details of these common photoreactive groups, as they have been previously discussed extensively in prior photoaffinity labeling reviews.[1–5] In particular, for a more thorough description of the chemistry, reactivity, labeling efficiency, selectivity, advantages, disadvantages, etc. of aromatic azides, diazirines, and benzophenones as photoreactive groups, the reader is encouraged to consult the most recent review with respect to photoaffinity labeling.[3]

1.2. Reporter groups

In order to ascertain and facilitate the isolation of photolabeled products, reporter groups such as radioactive isotopes (e.g., ^{125}I, ^{3}H), biotin, epitope tags (e.g., FLAG peptide), or fluorophores are often directly incorporated into the structure of the photoprobe. Radioactive tags are advantageous in that they are relatively small



Scheme 1. (a) Schematic of tandem photoaffinity labeling–bioorthogonal conjugation. Initially, a probe (yellow) possessing a photoreactive group and a 'clickable' handle/bioorthogonal chemical reporter is used to covalently label a target (orange) via photoaffinity labeling. A variety of tracers or tags can then be added to the photoprobe via highly specific bioorthogonal conjugation chemistry. (b) Specific bioorthogonal ligation chemistries typically used include a Staudinger–Bertozzi ligation (top) or a copper-mediated cycloaddition (bottom).

in size, highly sensitive, and easy to detect. However, they are also harmful, undergo relatively fast degradation, and require special handling. Biotin, epitope tags, and fluorophores allow easy enrichment, isolation, and/or detection of photolabeled products, but they can be relatively large in size, cell impermeable, and may negatively affect biological activity by sterically disrupting key interactions between the biological target and the photoprobe. Once again, it is beyond the scope of this report to further delineate the advantages, disadvantages, and details of these common reporter groups as they have been previously discussed extensively elsewhere. For a more thorough discussion of isotope, affinity, and fluorescent tags traditionally employed in proteomic studies, the reader is encouraged to consult a recent review.[13]

1.3. Tandem photoaffinity labeling–bioorthogonal conjugation

Towards addressing the significant disadvantages previously noted, a strategy has emerged wherein photoprobes are synthesized containing either a terminal alkyne or aliphatic azide as a 'clickable' handle/chemical reporter. Following covalent bond formation between the ligand and the biological target via photoirradiation (Step 1 in Scheme 1a), bioorthogonal conjugation reactions (Step 2 in Scheme 1a) such as the Staudinger–Bertozzi ligation[14] or copper-catalyzed Huisgen 1,3-dipolar cycloaddition ('click' reaction)[15] (Scheme 1b) are used to attach a tag of choice specifically to the aliphatic azide or terminal alkyne within the photoprobe. This two-step tandem photoaffinity labeling–bioorthogonal conjugation strategy is highly advantageous because aliphatic azides and terminal alkynes are relatively small, easily installable functional groups that are less likely to negatively influence biological activity by disrupting key ligand–target interactions. Additionally, these functional groups are biologically compatible and the approach allows for tag flexibility, high-throughput analysis, and use in vivo. The only apparent disadvantage of the tandem photoaffinity labeling–bioorthogonal conjugation strategy is that bioorthogonal chemical coupling yields (Step 2 in Scheme 1a) can vary in different systems. In particular, lower yields have been generally observed using Staudinger–Bertozzi ligation versus copper-catalyzed Huisgen 1,3-dipolar cycloaddition.[16] However, this variability in bioorthogonal conjugation yield appears to be significantly trumped by the noteworthy advantages associated with the strategy.

2. Applications of tandem photoaffinity labeling–bioorthogonal conjugation in medicinal chemistry

2.1. Affinity-based protein profiling (AfBPP)

Within the past decade, activity-based protein profiling (ABPP) via employment of irreversible chemical probes has surfaced as a powerful strategy to study the subcellular location and activities of receptors and enzymes in their natural environment. As a result, ABPP has emerged as a game-changing tool for drug discovery[17] and is often coupled with photoaffinity labeling.[3] A number of clickable ABPP probes bearing photoreactive groups have been previously discussed within smaller sections of larger reviews.[3,10,18] In particular, these clickable compounds are traditionally referred to as affinity-based probes (AfBPs), wherein the photoreactive group is used to facilitate covalent bond formation between the probe and an enzyme target that does not employ a catalytic nucleophilic amino acid within the ligand-binding site. This is in distinct contrast to that seen with traditional affinity labeling or mechanism-based enzyme labeling.[19] For example, photoreactive AfBPs bearing clickable handles have been used to profile many disease-relevant enzymes including galectins,[20–23] metalloproteases,[24–26] histone deacetylases (HDACs),[27,28] and type I methionine aminopeptidase.[29] Only the most recent examples of clickable, photoreactive AfBPs for protein profiling will be discussed.

2.1.1. AfBPs for carbohydrate-binding proteins

A number of probes for labeling and profiling carbohydrate-binding proteins via tandem photoaffinity labeling–bioorthogonal conjugation have emerged (Fig. 1). For example, galectin-3 is a β-galactoside-binding protein that interacts with a number of oncogenic proteins and cell cycle regulators. As a result, selective galectin-3 inhibitors have been pursued as potential anti-inflammatory agents and indirect inducers of apoptosis for the treatment of cancer. Towards better understanding the mechanisms and roles of galectin-3 in various disease states, high-affinity thiodigalactoside ligands (**1**) were synthesized containing either a benzophenone (e.g., **1a**) or acetophenone (e.g., **1b**) photoreactive group and a propargyl ether clickable handle.[30] In particular, specific galectin-3 labeling within a human cancer cell lysate was achieved by attaching aromatic photolabels to the 3-OH of carbohydrate **1**. Rational placement of the aromatic photolabels at this position greatly enhanced probe affinity to galectin-3 by facilitating π-cation interactions between the probe and select arginine residues. Interestingly, higher labeling efficiency and galectin-3 affinity was achieved when more flexible, less hydrophobic acetophenone derivatives were used compared to their more rigid, more hydrophobic benzophenone counterparts. In a similar manner, compounds based on the iminosugar deoxynojirimycin (**2**) were synthesized and pharmacologically evaluated as probes for labeling β-glucosidases, many of which are implicated in a number of diseases including Gaucher's disease.[31] In particular, alkynyl benzophenone probe **2a** showed very high potency (IC_{50} = 20 nm) in inhibiting non-lysosomal glucoceramidase and could specifically label this enzyme within a complex protein mixture.

Hosoya et al., provided one of the first examples of tandem photoaffinity labeling–bioorthogonal conjugation in medicinal chemistry.[32] This initial work featured creation of a diazide probe based on the cholesterol-lowering agent cerivastatin. The probe was subsequently used to label the catalytic domain of human HMG-CoA reductase, the well-established drug target for statins as cholesterol-lowering drugs. In particular, this pioneering cerivastatin photoprobe featured the parent ligand conjugated to a phenyl ring bearing an aryl azide photoreactive group and an aliphatic alkyl azide chemical reporter, which survives photolysis. The three substituents were spatially oriented in a 1,3,5-fashion around the aromatic phenyl ring. Additionally, parameters for this diazido motif have been recently described for application of designed photoprobes in molecular dynamics studies.[33] In turn, this and several other diazide structural motifs have become integral to a number of clickable photoprobes (vide infra).[32,34–41] For example, deoxynojirimycin diazide probe **3** was shown to specifically label *exo*-α-glycosidases within the complex proteome of an *Escherichia*

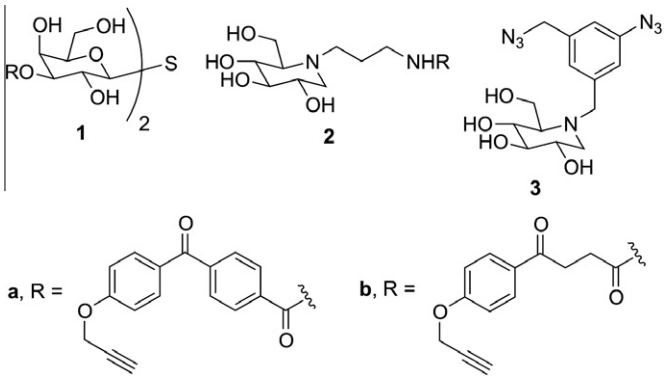


Figure 1. Clickable photoprobes for profiling carbohydrate-binding proteins.

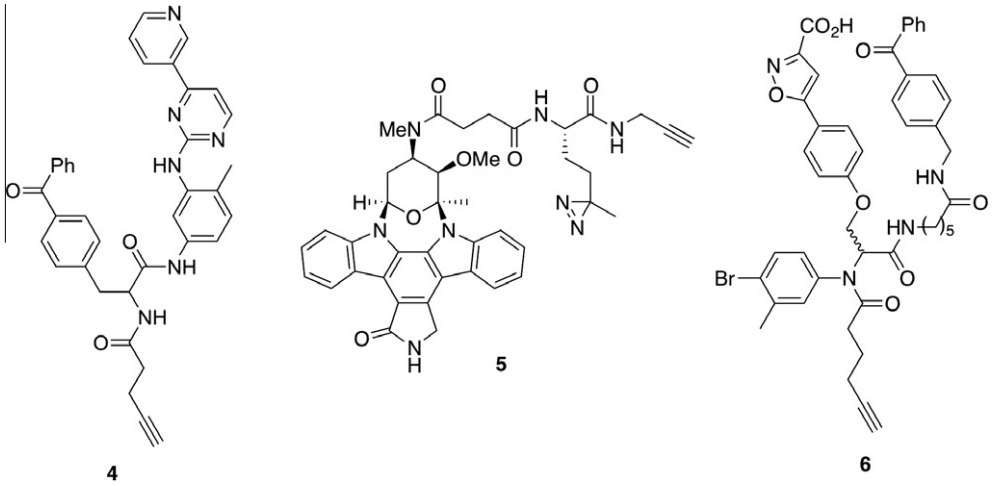


Figure 2. Clickable AfBPP photoprobes for kinases and protein tyrosine phosphatases.

coli lysate.[40] This particular work was somewhat unique in that it featured utilization of a phosphine-FLAG reagent during the Staudinger–Bertozzi bioorthogonal conjugation step (top reaction in Scheme 1b). The overwhelming majority of tandem photoaffinity labeling–bioorthogonal conjugation examples feature using either a clickable biotin or fluorophore reagent, some of which are commercially available, during the bioorthogonal conjugation step (Step 2 in Scheme 1a). It should be noted that aliphatic azide chemical reporters provide more options to the researcher during the bioorthogonal conjugation step versus alkyne counterparts. In theory, Staudinger–Bertozzi ligation, copper-catalyzed Huisgen 1,3-dipolar cycloaddition, or even strain-promoted click chemistry reactions[42] may potentially be used to facilitate the bioorthogonal conjugation step. However, several reports indicate higher levels of undesirable background protein adduction when alkynyl biotin or fluorophore reagents are used in bioorthogonal conjugation versus azide counterparts.[38,43,44] This latter observation may lead one to conclude that alkynes are perhaps more favorable chemical reporters for photoprobe development versus azide chemical reporters. In fact, survey of the tandem photoaffinity labeling–bioorthogonal conjugation chemical literature appears to support this conclusion, as aliphatic terminal alkynes are used more frequently than aliphatic azides as chemical reporters.

2.1.2. AfBPs for kinases and phosphatases

Given protein kinases are involved in the most prevalent post-translational mode of cellular signal transduction, dysregulation of kinase activity has been implicated in a number of diseases. In particular, there is a well-established connection between Abelson tyrosine kinase (Abl) activity and chronic myelogenous leukemia (CML). With this in mind, Imatinib-inspired photoprobe **4** (Fig. 2) was developed and showed highly specific, active-site-directed labeling of Abl kinase present in a crude mammalian proteome.[45] In particular, utilization of the tandem photoaffinity labeling–bioorthogonal conjugation strategy proved particularly advantageous in this case, as a derivative of **4** featuring alkyne replacement with a bulky rhodamine fluorophore led to poor labeling efficiency and specificity. Photoprobe **4** displays a number of promising characteristics and may serve as a valuable compound for in situ screening of Abl inhibitors.

L-Photo-methionine-based probe **5** represents the first cell-permeable kinase probe capable of proteome-wide profiling of potential cellular targets of staurosporine, a natural product regarded as one of the most potent general protein kinase inhibitors.[46] Once again, application of the tandem photoaffinity labeling–bioorthogonal conjugation strategy proved necessary, as a previously reported staurosporine-derived photoprobe bearing a biotin tag (i.e., for photoaffinity biotinylation[47]) proved too bulky to enter cells.[48] In particular, the authors noted that the choice of a small alkyl diazirine over a bulkier benzophenone photoreactive group, as well as the alkyne handle, was crucial in keeping the overall probe small and cell-permeable. Furthermore, molecular docking experiments were performed with **5** in the ATP site of protein kinase A to ensure the extra groups introduced did not affect binding to at least one of staurosporine's known kinase targets. Photoprobe **5** proved valuable in proteome-wide profiling of potential cellular targets of staurosporine in live HepG2 cells, thus suggesting the tandem photoaffinity labeling–bioorthogonal conjugation strategy could be generally applied to develop other kinase probes in the future.

Likewise, dysregulation of protein tyrosine phosphatases (PTP) in phosphoproteome networks has been implicated in a plethora of human disease states including diabetes and obesity. Photoprobe **6** is somewhat unique in that it was discovered as a member of a small library of 25 AfBPs rapidly synthesized via a multicomponent Ugi reaction using an isonitrile-containing benzophenone.[49] In particular, probe **6** and other members of the library were rationally explored based on the structural properties of previously developed bidentate PTP inhibitors featuring a phosphotyrosine mimic connected to a diversity group through a suitable linker. The probes showed good labeling of PTP1B and MptpB, and were subsequently used to label endogenous PTP1B in MCF-7 cell lysates.

2.1.3. AfBPs for ligand-gated ion channels

For the most part, ABPP has been extensively used to characterize the physiological functions of enzymes. However, Tantama and co-workers vertically advanced the field of ABPP by applying the tandem photoaffinity labeling–bioorthogonal conjugation strategy to profile a ligand-gated ion channel.[50] BPyneTEA (**7**, Fig. 3) represents a state-dependent photoaffinity probe for the nicotinic

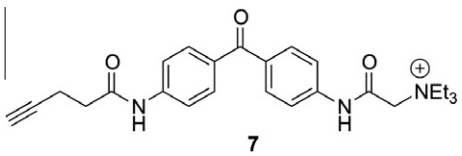


Figure 3. BPyneTEA (**7**) functions as an effective ABPP probe for the nicotinic acetylcholine receptor, a ligand-gated ion channel.

acetylcholine receptor (nAChR). In particular, probe **7** may be useful for studying the neurobiology of nAChR desensitization associated with neuromuscular disorders and nicotine addiction. In living cells, BPyneTEA photolabeled the closed conformation of the nAChR ion channel selectively over the inactive desensitized state. Given there are a host of well-characterized state-selective blockers for NMDA receptors, 5-HT_3 receptors, and potassium channels, it is plausible ion channels may represent generally useful targets for ABPP via the tandem photoaffinity labeling–bioorthogonal conjugation approach.

2.2. Identification of drug target(s)

The identification of specific biological targets associated with a particular disease is the starting point for modern drug discovery and development. In particular, application of the tandem photoaffinity labeling–bioorthogonal conjugation strategy has significantly accelerated the challenging endeavor of identifying protein-binding partners for bioactive small molecules. Furthermore, coupling molecular modeling with photoaffinity labeling and LC–MS proteomics can aid in elucidating the 3-D structure of a ligand–target complex at the molecular level (binding ensemble profiling, vide infra). Several clickable photoprobes used to identify the protein targets of bioactive small molecules have been previously discussed in smaller sections of larger reviews.[3,10] These include identification of HDAC3 as the target for pimelic diphenylamide inhibitors as promising therapeutics for Huntington's disease and Friedreich's ataxia,[51] and the LasR receptor protein as the binding partner for *Pseudomonas aeruginosa* quorum sensing compound *N*-(3-oxododecanoyl) homoserine lactone.[52] Only the most recent examples of clickable photoprobes for biological target identification will be discussed.

2.2.1. Clickable azide-based photoprobes for drug target identification

Returning to synthetic amino acid-based compounds, serine-derived aryl azide **8a** (Fig. 4) was synthesized and pharmacologically evaluated in vivo as a potential photoprobe for identifying the drug target(s) of lacosamide, a first-in-class anti-epileptic drug approved for adjunctive treatment of partial-onset seizures in adults.[53] In spite of a two-fold improvement in anticonvulsant activity seen with aryl azide photoprobe **8a** versus isothiocyanate affinity label **8b**, the isothiocyanate probe proved superior to **8a** in revealing potential lacosamide binding targets within a mouse brain-soluble proteome. The authors concluded the differences in chemical reactivity between the photoreactive aryl azide and electrophilic aryl isothiocyanate was a critical factor for successful target detection, particularly since lacosamide's binding is modest. Furthermore, this work suggests researchers should explore both photoaffinity and affinity labeling approaches in target identification programs, given the inherent differences in chemical reactivity between photoreactive groups and traditional electrophiles, as well as consideration of whether the probes will be used in vivo or in vitro.

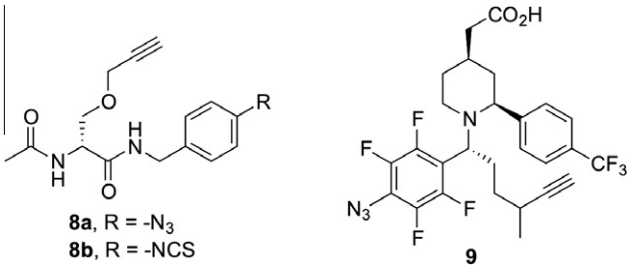


Figure 4. Azide-based clickable probes for drug target identification.

With respect to Alzheimer's disease (AD), γ-secretase modulators (GSMs) are being developed as potential AD therapeutics given their ability to reduce formation of Aβ 42, a pathogenic peptide that aggregates and deposits within the brain of AD patients. Tandem photoaffinity labeling–bioorthogonal conjugation was used to resolve the binding partner of a lead piperidine acetic acid GSM.[54] In particular, perfluorinated aryl azide analog **9** identified presenilin-1 (PS1) as the drug target for the lead compound within the γ-secretase complex. Further experiments with the photoprobe provided evidence that allosteric interaction of GSMs with PS1 modulates secretase activity via conformational change of the active site within the γ-secretase complex.

2.2.2. Clickable benzophenone-based photoprobes for drug target identification

Despite being relatively large and hydrophobic, survey of the click chemistry literature indicates benzophenones as the most commonly employed photoreactive group in target discovery programs. For example, hypoxia-inducible factor (HIF) inhibitors possess anti-angiogenic activity, wherein such compounds may prove beneficial as cancer chemotherapeutics. Ban et al. manipulated *o*-carboranylphenoxyacetanilide, a potent HIF-1α inhibitor, into two clickable benzophenone-based photoprobes (**10**, Fig. 5) in order to elucidate the primary binding proteins and mechanism of action of the parent compound.[55] Using tandem photoaffinity labeling–bioorthogonal conjugation, heat shock protein 60 (HSP60) was identified as the primary target protein of *o*-carboranylphenoxyacetanilide, and further experiments provided evidence that HSP60 affects HIF-1α accumulation.

Tandem photoaffinity labeling–bioorthogonal conjugation was also applied to unravel the non-canonical protein targets of vancomycin, a clinically significant antibiotic wherein bacterial resistance represents a tremendous threat to human health. Probes such as **11** were shown to label autolysin Atl and ABC transporter protein in living *Staphylococcus aureus* and *Enterococcus faecalis* cells, respectively.[56] As a result, further exploration of the vancomycin-binding mode at these particular proteins may allow for rational design of analogs with improved clinical properties.

Despite the noted advantages of the tandem photoaffinity labeling–bioorthogonal conjugation approach, it is well known that non-specific labeling of various proteins, especially abundant and sticky proteins, as well as actual target proteins, has been a major problem with respect to photoaffinity labeling in drug target discovery programs. Towards addressing this limitation, a new method called fluorescence difference in two-dimensional gel electrophoresis (FITGE) was developed to observe the interactions between proteins and small molecules in an intact cellular environment.[57] With the use of bioactive photoprobe **12** in parallel with inactive clickable photoprobe **14**, FITGE proved effective over conventional methods by successfully identifying the protein target of an anti-proliferative compound (**13**) in live cells through the differentiation between specific and extensive non-specific binding of the photoaffinity probes. In short, the method is inspired by difference gel electrophoresis, wherein active-probe-labeled and negative-probe-labeled proteomes are crosslinked and conjugated to different fluorophores, pooled into a single sample, then separated by two-dimensional gel electrophoresis (2DGE). As a result, non-specific binding events can be readily ruled out by direct comparison of the labeling patterns of the whole proteomes in 2DGE. Following fluorescent gel image analysis, the desired protein spots from the active probe are cut out and subjected to mass analysis for identification of potential target proteins. In particular, probe **12** was used to confirm that parent compound **13** functions as a potent antitumor agent by inhibition of tubulin polymerization.

Additionally, pretubulysins have been described to retain the high tubulin degradation activity of their more complex tubulysin

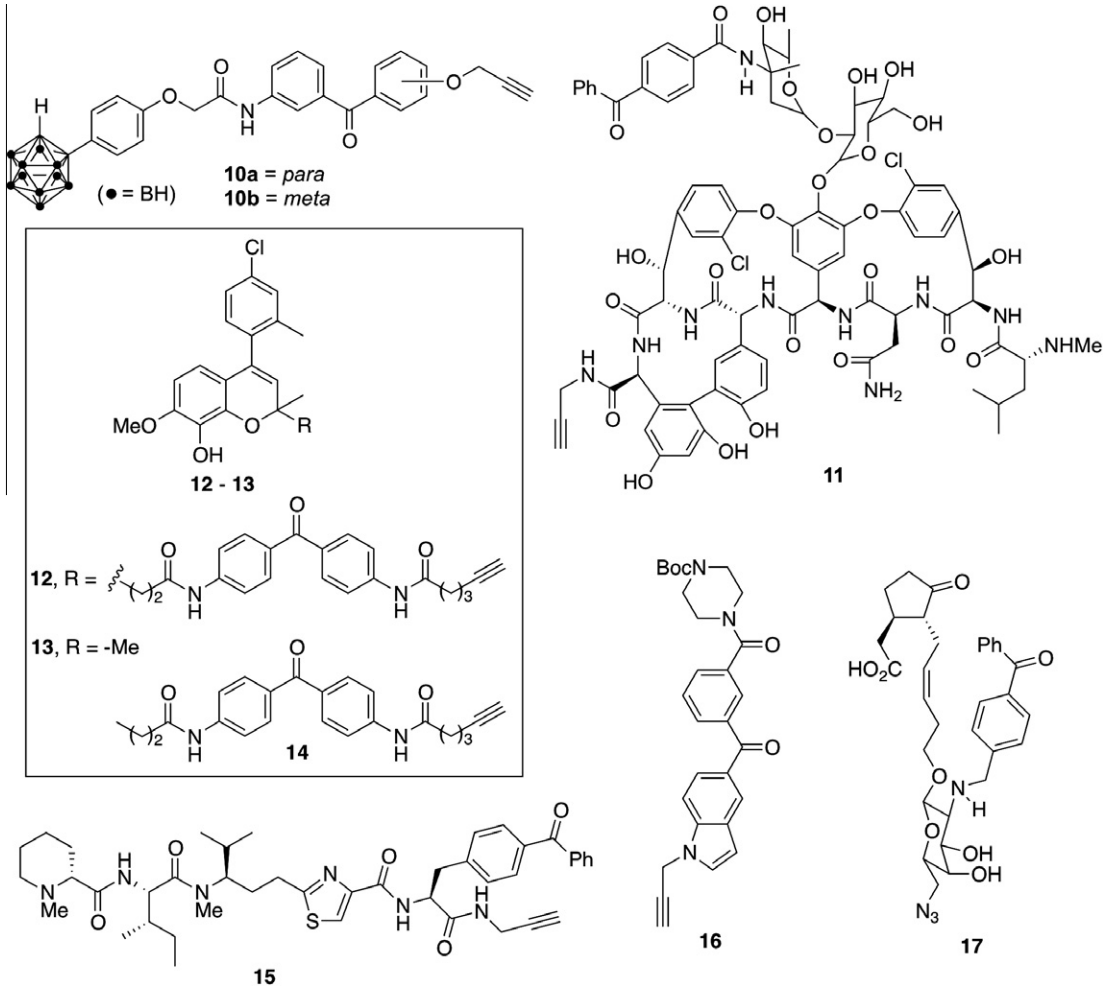


Figure 5. Benzophenone-based clickable photoprobes for target identification.

relatives, yet they are significantly easier to synthesize. Although, tubulin has been suggested as the molecular target for tubulysin, a comprehensive target analysis employing pretubulysin-based probe **15** was conducted and indicated that pretubulysin displays rather remarkable selectivity for beta-tubulin.[58] Additionally, probe **15** was used to visually monitor tubulin binding in cells and its associated inhibition of microtuble assembly.

Promising compounds that result from phenotypic screening programs often tend to interact with multiple protein targets with moderate to weak affinities. As a result, it is difficult to characterize the interactions of these compounds in living systems as a means of understanding the mechanistic basis behind their pharmacology. In order to address this challenge, Cisar and Cravatt have recently put forth a chemoproteomic strategy that can be combined with cell-based screening to enable global and quantitative assessment of reversible small molecule-protein interactions directly in living systems.[59] In particular, the strategy features synthesis and utilization of compounds bearing a photoreactive group and clickable handle for application of tandem photoaffinity labeling–bioorthogonal conjugation after phenotypic screening. Compound **16** from a small library of clickable, photoreactive compounds was found to inhibit cancer cell proliferation selectively under nutrient-limiting conditions via phenotypic screening, and MT-ND1, an integral membrane subunit of NADH/ubiquinone oxidoreductase complex 1, was identified as the specific target for the probe. This work provides proof-of-principle that direct generation and integration of clickable photoprobes into phenotypic screening programs should facilitate the discovery of bioactive probes, which are further amenable to mechanistic characterization and accelerated target identification using advanced chemical proteomic techniques.

Finally, as an example of a benzophenone-based probe bearing an aliphatic azide clickable handle, compound **17** was synthesized and evaluated in order to identify the membrane target protein of jasmonateglucoside.[60] In this particular case, application of the tandem photoaffinity labeling–bioorthogonal conjugation technique demonstrated lower labeling efficiency relative to a biotinylated jasmonateglucoside photoprobe bearing a biaryl linker synthesized by click chemistry. The biotinylated photoprobe was shown to adopt a unique T-shape conformation that raised labeling efficiency relative to the stepwise tagging approach using probe **17**. This example indicates that in some cases photoaffinity biotinylation[47] produces more desirable results versus tandem photoaffinity labeling–bioorthogonal conjugation, thus suggesting researchers should explore both approaches during the course of their work.

2.2.3. Clickable diazide-based photoprobes for drug target identification

As previously exemplified, tandem photoaffinity labeling–bioorthogonal conjugation can represent a very powerful strategy to identify the biological targets of complex natural products. An additional case in point features diazirine-alkyne probe **18b** (Fig. 6), which was designed by replacement of particular amino acid residues within HUN-7293 (**18a**), a fungal cyclodepsipeptide

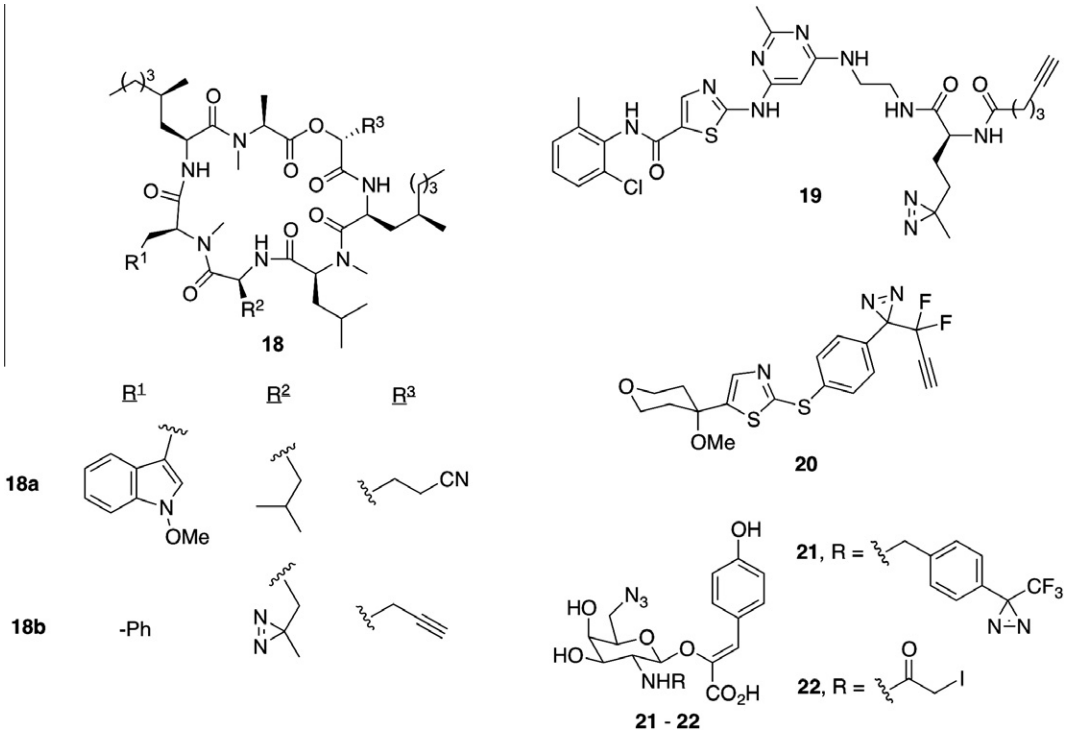


Figure 6. Diazirine-based clickable probes for target identification.

inhibitor of cotranslational translocation.[61] Specifically, a leucine (R^2) and nitrile-based residue (R^3) within a phenylalanine (R^1) derivative of HUN-7293 were replaced with photo-leucine and a terminal alkyne, respectively. Tandem photoaffinity labeling–bioorthogonal conjugation then enabled identification of Sec61α, the structural core of the Sec61 translocation channel, as HUN-7293's target.

Photoprobe **19** is based on the dual Src/Abl kinase inhibitor Dasatinib (BMS-354825), which has been used for the treatment of Imatinib-resistant CML. In an effort to identify additional cellular targets associated with Dasatinib anti-cancer activity, tandem photoaffinity–bioorthogonal conjugation was applied.[62] By comparing results from different proteomic set-ups (live cells, cell lysates, and immobilized affinity matrix), probe **19** was able to identify significantly more putative kinase targets previously unknown to Dasatinib by maintaining the compound's highly regulated, compartmentalized kinase–drug interactions. In turn, the unknown Dasatinib targets were further validated by pull-down/immunoblotting experiments as well as kinase inhibition assays.

Clickable photoprobe **20** is rather distinct in that it contains an 'all-in-one' 3-(1,1-difluroprop-2-ynyl)-3*H*-diazirin-3-yl functional group.[63] This compact structural unit was rationally designed and directly incorporated into a lead compound with the purpose of indentifying proteins involved in the biosynthesis of leukotrienes as inflammation and asthma mediators. Although, no proteomic studies were reported for this photoprobe, proof-of-concept studies were performed in MeOH resulting in isolation of a methoxy ether adduct upon photoirradiation, thus indicating the highly unstable carbene that results from diazirine photodecomposition does not react with its neighboring acetylene moiety. Subsequent click reaction of the ether adduct with an azido biotin derivative proceeded smoothly resulting in the expected triazole conjugate.

As an example of a diazirine photoprobe bearing an azide clickable handle, probe **21** was synthesized and evaluated in an effort to

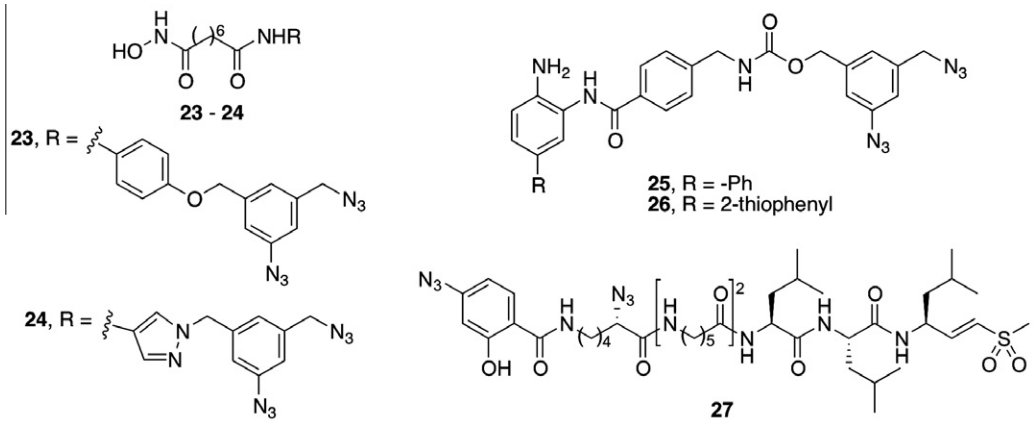


Figure 7. Diazido-based clickable photoprobes for BEProFL studies.

identify the cytosolic target protein of potassium isolespedezate.[64] In this case, utilization of affinity probe **22** in a stepwise fashion with an alkynyl FLAG tag proved more successful than the photoaffinity approach, resulting in nanogram-scale purification and identification of MetE as the target.

2.3. Binding ensemble profiling with (f)photoaffinity labeling (BEProFL)

In order to obtain valuable information for structure-based drug design and validate/refine molecular models of biological targets, tandem photoaffinity labeling–bioorthogonal conjugation can be coupled with LC–MS and molecular modeling studies in order to determine the 3-D structure of a ligand–target complex. In particular, combining these powerful experimental approaches facilitates identification of key ligand–target binding interactions and conformational preferences for both the ligand and biological target. Utilization of this merged approach to map the binding sites and poses of ligands within biological targets has been termed BEProFL (binding ensemble profiling with (f)photoaffinity labeling).[38] In addition to guiding ligand optimization, BEProFL is expected to validate and refine computer-aided drug discovery methods such as virtual/in silico screening.

2.3.1. Clickable azide-based photoprobes used in BEProFL studies

With the aim of gaining information for future drug design of isoform-selective compounds, BEProFL has emerged as a method towards understanding the binding modes of HDAC inhibitors as epigenetic modulators. Initial studies in this area featured utilization of photoprobe **23** (Fig. 7), which was shown to modify HDAC8 at or near Asp^{233} and Asp^{272} by adopting at least two different binding modes consistent with molecular dynamics simulations.[38] Subsequent work featured docking studies with isoxazole- and pyrazole-based compounds, indicating the former chemotype is not flexible enough to occupy a second binding site within HDAC8.[34] In particular, pyrazole-based compound **24** was found to be a very active HDAC8 inhibitor (IC_{50} = 17 nM). Further pharmacological studies with the isoxazole- and pyrazole-based compounds indicated the diazide functionality did not interfere with neuroprotection properties or cell permeability. In more recent studies, two benzamide scaffolds were successfully explored for the design of novel HDAC2 probes suitable for BEProFL.[65] In particular, the most active probes were compounds **25** and **26**, which have an IC_{50} of 0.9 and 1.2 μM, and 300 and 350 nM, for HDAC1 and 2, respectively. This represents an ~30-fold selectivity for HDAC1 and 2 relative to HDAC3 and 8.

An additional example of a diazido probe used in a BEProFL approach is tripeptide **27**, which is multifunctional in that it bears a photoreactive aryl azide, a vinyl sulfone electrophilic trap, and an aliphatic azide bioorthogonal tag for Staudinger–Bertozzi ligation. Compound **27** was part of a series of probes designed to investigate how compounds are positioned within a proteosome cavity, particularly with respect to probe interactions of residues at positions distal to the active site-reactive group.[41] The biochemical approach for probe **27** is rather unique in that it features a multi-step sequential protocol of affinity labeling, photoaffinity labeling, and bioorthogonal conjugation followed by LC–MS/MS. As a result of the first two steps generating two covalent bonds between the probe and the biological target, a more definitive and conformationally restricted ligand–target complex is expected. As a result, this noteworthy approach has been suggested as justifiable alternative to existing methods in biochemical research (e.g., X-ray crystallography) for determining the mode of action and orientation of a ligand within a binding site.

2.3.2. Clickable benzophenone-based photoprobes used in BEProFL studies

In order to probe the active site microenvironment of fatty acid amide hydrolase (FAAH), a drug target implicated for development of pain therapeutics, photoprobe **28** (Fig. 8) was synthesized as part of a series of clickable compounds to explore FAAH–substrate interactions in native cell membranes.[66] By varying the length of the linker between the carbamate and the benzophenone, data was generated that supported selective intermolecular crosslinking to a small-molecular weight product for longer photoprobes that were modeled to protrude from the FAAH active site.

Finally, BEProFL has been suggested as a confident method to map sites of protein–protein interactions.[67] As a test case example, photoprobe **29** was used to study the interaction between rhodopsin, a G-protein-coupled receptor, and transducin, its G-protein. Both of these proteins are crucial in the visual process. In particular, probe **29** is unique in that it bears a *p*-ethynylbenzoyl-modified phenylalanine residue, featuring the alkyne tag directly attached to an aromatic ring. Traditionally, clickable affinity- and photoaffinity labeling probes tend to employ aliphatic terminal alkynes as chemical reporters, presumably due to their faster reaction rates and milder reaction conditions during bioorthogonal conjugation. However, given the success of probe **29** in elucidating the protein–protein interaction between transducin and rhodopsin via tandem photoaffinity labeling–bioorthogonal conjugation, *p*-ethynylbenzoyl-modified phenylalanine represents a valuable contribution to the growing arsenal of photoreactive amino acids.

2.4. Studying endogenous biological molecules and imaging applications

Perhaps more from a biochemistry than a medicinal chemistry perspective, tandem photoaffinity labeling–bioorthogonal conjugation has been extensively used to explicate protein–lipid binding interactions and other biological roles of phospholipids.[68] Nonetheless, this area of research is very significant to the medicinal

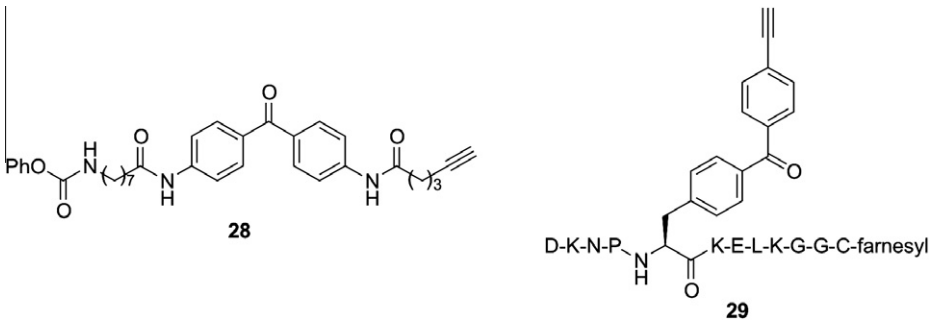


Figure 8. Benzophenone-based clickable photoprobes for BEProFL studies.

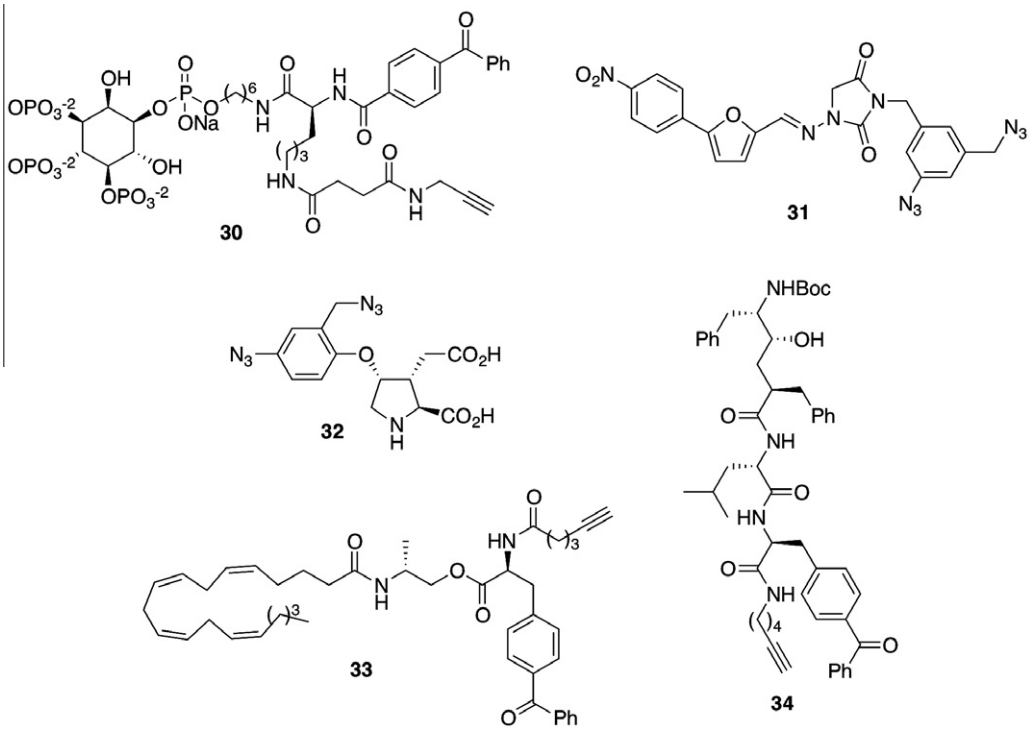


Figure 9. Clickable photoprobes for studying endogenous biological molecules and imaging applications.

chemistry community given lipids play a vital role in regulating many vital biological pathways. In particular, deviations in the signaling properties of lipids has been linked to a plethora of debilitating diseases. Clickable phosphatidylcholine-,[39] phosphatidic acid-,[69] and phosphatidylserine-based[70] photoprobes have been previously described in this context. Most recently, clickable phosphatidylinositol 3,4,5-triphosphate-based photoprobes (e.g., **30**, Fig. 9) have been developed to identify and characterize protein-binding partners of this lipid within cancer cell extracts.[71]

In an effort to elucidate the molecular mechanism of skeletal muscle contraction, a series of bifunctional dantrolene derivatives was synthesized for radioisotope-free photoaffinity labeling studies.[35] In particular, probe **31** (i.e., GIF-0430) was shown to selectively inhibit physiological Ca^{+2} release from the sarcoplasmic reticulum in mouse skeletal muscle without a strong effect on Ca^{+2}-induced Ca^{+2} release. Subsequent proteomic studies utilized a fluorescein-anchored triarylphosphine derivative in order to detect candidate target proteins for this probe via Staudinger–Bertozzi ligation. Likewise, diazido acromelic acid analog **32** was synthesized in order to study kainoid receptors, which are responsible for mediating powerful neuroexcitatory activity within the mammalian central nervous system.[36]

Finally, benzophenone-based probe **33** was developed to study cannabinoid (CB) receptors, which have been linked to pain, inflammation, neurodegeneration, and cognition.[72] In particular, compound **33** features a methanandamide moiety conjugated to the carboxy terminus of a clickable, photoreactive phenylalanine derivative. However, in this particular case, probe **33** proved inferior to a non-photoreactive, biotinylated derivative of 2-arachidonyl glyceryl ether in terms of direct visualization of CB_1 receptors in cells. Likewise, active site-directed photoaffinity probe **34** proved to be cell penetrant, thus representing a potentially useful tool for imaging γ-secretase upon conjugation to any number of azide-linked fluorescent reporters.[73]

3. Additional 'all-in-one' chemical moieties for tandem photoaffinity labeling–bioorthogonal conjugation

Apart from the examples previously discussed, there are a number of additional 'all-in-one' moieties and chemical reactions worth noting that are useful for readily arming a parent ligand with a photoreactive group and click chemistry handle. In addition to the size and conformational flexibility of these moieties (e.g., the amino acid alkynyl benzophenone moiety in probe **33** is significantly larger and more conformationally flexible versus the compact diazide moiety found in probe **31** (Fig. 9)), the number of synthetic steps needed to access these entities can be drastically different. For example, the alkynyl benzophenone reagent for

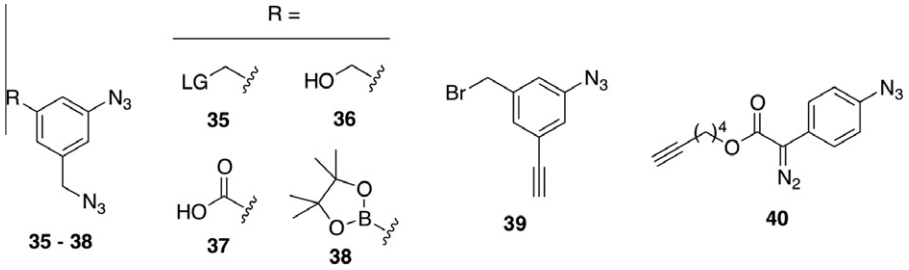


Figure 10. Examples of 'all-in-one' moieties for readily arming parent ligands with a photoreactive aryl azide and click chemistry handle.

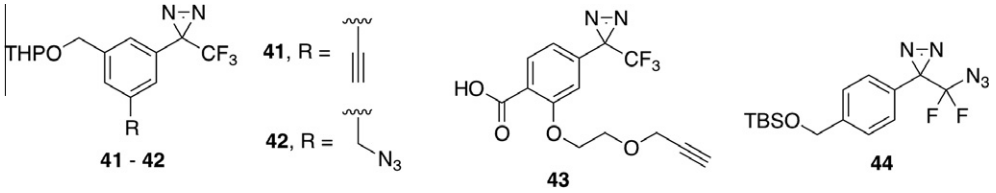


Figure 11. Examples of 'all-in-one' moieties for readily arming parent ligands with a photoreactive aryl diazirine and click chemistry handle.

arming parent compound **2** (Fig. 1) can be rapidly made in three steps from commercially available materials,[30] whereas assembly of alkynyl aromatic diazirine carboxylic acid **43** (Fig. 11) requires nine synthetic steps prior to arming a parent ligand.[75]

3.1. Photoreactive aryl azide-based 'all-in-one' moieties

Nucleophilic substitution of **35** (Fig. 10) bearing a benzyl leaving group (LG = bromide,[35,40] mesylate,[38] or tosylate[34]) represents the most common way for chemists to readily arm a parent ligand with the 1,3,5-trisubstituted phenyl motif bearing an aryl azide photoreactive group and methylene aliphatic azide clickable handle. However, an alkoxide variant of **36** has been reported as a nucleophile within a Williamson ether synthesis strategy, thus indicating the two azides within this structural motif can tolerate the relatively basic conditions traditionally associated with such a reaction.[32] More recently, a carboxylic acid variant (**37**) has been prepared allowing direct coupling to a parent ligand nucleophile.[34] Additionally, 3-azido-5-(azidomethyl)phenylboronic acid pinacol ester (**38**) has been described as a valuable reagent for synthesizing diazido-functionalized biaryl compounds via Suzuki–Miyaura coupling.[37]

Benzyl bromide reagent **39** is somewhat unique in that the terminal alkyne click chemistry handle is directly attached to the aromatic ring (i.e., analogous to that previously discussed for probe **29** (Fig. 8)). Support for using this motif in tandem photoaffinity labeling–bioorthogonal conjugation stems from the observation that 1-azido-3-ethynylbenzene undergoes photoreaction cleanly to give a didehydroazepine via an expected phenylnitrene species. In particular, bromide **39** was displaced by a dantrolene derivative analogous to the synthesis of probe **31** (Fig. 9).[35] Finally, diazo reagent **40** proved useful in arming alcohol-containing natural products via an O–H insertion reaction.[74] In particular, gibberellic acid methyl ester was reacted with **40** in the presence of catalytic $Rh_2(OAc)_4$ to give 57% yield of a mixture of conjugates stemming from O–H insertion at the 2° and 3° alcohol positions within the natural product. This example indicates both the aliphatic alkyne and aromatic azide are stable under the rhodium-catalyzed O–H insertion conditions.

3.2. Photoreactive aryl diazirine-based 'all-in-one' moieties

Once again, tetrahydropyranyl diazirine-based moiety **41** (Fig. 11) is unique in that the alkyne click chemistry handle is directly attached to the aromatic ring. Support for using this moiety in tandem photoaffinity labeling–bioorthogonal conjugation was provided by model reactions wherein the diazirine was first photoreacted under methanolic conditions, then clicked to benzyl azide to provide the expected methoxy-substituted triazole conjugate in 47% yield.[35] Likewise, similar model photoreactions were reported for aliphatic azide chemical reporter variant **42** under CD_3OD conditions.[32]

Carboxylic acid **43** represents another 'all-in-one' building block for arming parent ligands for tandem photoaffinity labeling–bioorthogonal conjugation applications. In particular, acid **43** was synthesized and coupled to bafilomycin A_1, a natural product macrolactone which functions as an inhibitor of V-ATPase. Additionally, model photo- and click chemistry reactions were reported for the isopropyl ester derivative of **43**.[75] Finally, compound **44** contains a unique 3-azidodifluoromethyl-3*H*-diazirin-3-yl group as a compact 'all-in-one' functional group for radioisotope-free photoaffinity labeling.[76] Analogous to the moiety within compound **20** (Fig. 6), the purpose of the difluoro substituents is to stabilize the adjacent carbene that results upon photoirradiation. Once again, proof-of-principle studies for tandem photoaffinity labeling–bioorthogonal conjugation were performed with this moiety. First, diazirine photodecomposition in methanol using 365 nm UV light provided the corresponding methyl ether adduct as the major product. The aliphatic azido group was left intact under these conditions and was subsequently coupled to phenylacetylene under copper-catalyzed click chemistry conditions.

4. Conclusions

In summary, photoaffinity labeling has undergone significant advancement over the past decade due to its coupling with bioorthogonal/click chemistry reactions. In particular, tandem photoaffinity labeling–bioorthogonal conjugation has proved valuable to medicinal chemists and chemical biologists via a number of applications including affinity-based protein profiling, target identification, binding ensemble profiling, studying endogenous biological molecules, and imaging applications. Furthermore, the development of this chemical approach over the past decade has served to provide these researchers with a number of 'all-in-one' structural units that are available to arm a parent ligand of choice with a photoreactive group and click chemistry handle. Given the timelessness of photoaffinity labeling as a standard biochemical technique and the continued emergence of new bioorthogonal/click chemistry reactions, it is anticipated that coupling these chemical avenues will continue to be explored in medicinal chemistry and chemical biology for years to come.

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