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Photolytic Labeling and Its Applications in Protein Drug Discovery and Development

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Abstract

In this mini-review, the major types of photolytic labeling reagents are presented together with their reaction mechanisms. The applications of photolytic labeling in protein drug discovery and development are then discussed; these have expanded from studies of protein-protein interactions *in vivo* to protein-matrix interactions in lyophilized solids. The mini-review concludes with recommendations for further development of the approach, which include the need for new and more chemically diverse photo-reactive reagents and better understanding of the mechanisms of photolytic labeling reactions in various media.

Keywords

photolytic labeling; protein drug; phenyl azide; carbene; diazirine; benzophenone; protein-protein interaction; lyophilized solid

Introduction

Photolytic labeling, also known as photoaffinity labeling and photo-crosslinking, is a series of chemical reactions that are activated upon exposure to light at a certain wavelength. With covalent bond formation after UV activation, transient protein-protein or protein-ligand interactions can be captured.[1,2] The use of photo-reactive reagents can be dated back to 1969 when the 4-azido-2-nitrophenyl group was first used to label bovine γ-globulin and human serum albumin to study antibodies *in vivo*[3] Today, the application of photolytic labeling has been expanded to several areas including the identification of membrane protein targets, the elucidation of protein structure in solution and the characterization of proteins in pharmaceutical solids.[4–6] Photo-reactive reagents typically are stable before UV activation and become reactive under specific activation conditions. Moreover, photolytic labeling generates covalently modified products that are stable enough to be analyzed with common analytical methods. These properties have allowed photolytic labeling to be increasingly used to study protein interactions both *in vitro* and *in vivo*.

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Selecting appropriate photo-reactive reagents has been important in the success of photolytic labeling studies. In this mini-review, the reaction mechanisms of common photo-reactive reagents will be introduced and the reported applications of these reactions in the discovery and development of protein drugs will be reviewed. The applications include studies of protein-protein interactions, protein-ligand binding, structure of the native protein and its oligomers, and protein-matrix interactions in lyophilized solids.

Photo-Reactive Reagents

1. Phenyl Azide

Phenyl azide labels have been incorporated in the protein structure to study protein-protein interactions, often using modified amino acids such as p-azido-L-phenylalanine.[7] The absorption maximum for the phenyl azide group is ~260 nm when the phenyl ring is unsubstituted.[8] In studies involving proteins, the short wavelength required to activate phenyl azides may cause damage to protein structures.[9] The reaction mechanism has been studied in a rigid glassy system consisting of diethyl ether, isopentane and ethanol at 77K.[10] After UV activation, singlet and triplet phenylnitrene were proposed as transient intermediates. Phenylnitrene can insert into C-H, N-H or O-H bonds and form covalently labeled products. Phenylnitrene can also undergo ring expansion to form addition products with nucleophiles such as primary or secondary amines. Trace amounts of azobenzene were detected as side products of the reaction.[10] The oxidation of phenylnitrene with formation of nitrobenzene has also been reported.[11] The reaction products can be affected by system temperature and by substituent groups on the aromatic ring.[12] With many possible reaction pathways (Fig. 1), the labeling yields are often low after irradiation.[13] Nitrenes have shown different reactivities towards naturally occurring amino acids, with a preference for cysteine and aromatic amino acids, suggesting that labeling may favor interactions involving these amino acids.[14]

2. Carbene

Carbenes can form covalent bonds with molecules in their vicinity by inserting into C-C bonds, X-H (X=C, O, N, S) bonds or adding onto C=C bonds.[15–17] Photolytic labeling with carbenes is a fast, relatively non-specific radical reaction. Carbenes can be generated from diazirine or diazo groups after UV activation at a certain wavelength.[18] Stable diazo compounds such as diazo ketones have been reported to undergo the Wolf rearrangement (see Fig. 2B), which leads to the formation of ketene products.[19] Ketenes can be further hydrolyzed by water to form carboxylic acids,[20] or can react with other nucleophiles. The inherent instability and low labeling yield of diazo compounds has limited their application in photolytic labeling studies.[8]

Unlike diazo compounds, diazirines tend to be relatively stable, allowing their broad application in protein interaction studies.[21] In the absence of activating light, diazirines are stable at room temperature. Activation requires absorption of light at relatively long wavelengths (330-370 nm), causing less damage to protein structure than with phenyl azides. It has been shown that the diazirine radical reaction also occurs in amorphous solid powders.[22] In addition to the expected photolytic labeling products, diazirine adducts with

water and phosphate from buffer salt were detected in solution and solid-state reactions in these studies.[23] "Dead-end" products, resulting from deactivation of the carbene without intermolecular reaction, accounted for a large fraction of the photolytic labeling products in these samples.[23] The structures of these dead-end products remain unknown. Ketones can form at the carbene carbon after oxidation by O_2 or hydrolysis of N-H insertion products.[16] Photolysis of the diazirine can induce conversion to diazo compounds, which have low reactivity and can lead to undesired side products.[24] A trifluoromethyl group can be introduced into the diazirine carbon to stabilize the carbene and prevent rearrangement.[25] The reaction pathways of diazirines are shown in Fig. 2, using trifluoromethylphenyldiazirine (TFMD) as an example.

The selectivity of the carbene towards different amino acid side chains has been studied in fiberglass coupled with 3-(trifluoromethyl)-3-(m-isothiocyanophenyl) diazirine.[26] In this study, the carbene preferentially interacted with cysteine and aromatic amino acids while relatively low affinity was observed for amino acids with aliphatic side chains. Although carbene reactions are thought to be relatively non-specific in solution, it remains unclear whether the preferential reactivity observed in the fiberglass system also occurs in amorphous powders containing proteins.

3. Benzophenone

Benzophenones have also been used as photo-reactive reagents in protein interaction studies. For example, p-benzoyl-L-phenylalanine (pBpA) has been incorporated into proteins *in vitro* and *in vivo* as a photo-reactive analog of phenylalanine to study protein-ligand binding and protein-protein interactions.[27–29] Benzophenone can be activated with UV light at 350–360 nm to form a reactive ketyl radical that reacts preferentially with C-H bonds. A new C-C bond forms between the ketone carbon from benzophenone and the carbon from the C-H bond (see Fig. 3).[30] C-H bonds adjacent to N or S atoms, especially those in methionine, are favored reaction sites for benzophenone compounds.[31–33] Proteins are less damaged at the wavelengths required for the activation of benzophenone than with the shorter wavelengths needed for phenyl azides, although benzophenone occasionally requires a relatively long irradiation period.[34] The structure of benzophenone makes it stable in many solvents. However, one of the disadvantages of using benzophenone is that its structure does not mimic those of natural amino acids without aromatic rings. As a result, protein structure may be disrupted with the incorporation of pBpA or other benzophenones.

4. Other Reagents

Click-chemistry is an addition reaction with high specificity. A typical example is an azide-alkyne cycloaddition to form triazole compounds with copper as a catalyst.[35] While the high specificity may be desirable in some cases, the requirement of metal ions may not be suitable for protein interaction studies. A metal-free click chemistry reaction has been achieved in human Jurkat cells.[36] N-azidoacetyl-sialic acid was metabolically introduced into cell surface glycoproteins. Biotinylated cyclopropenone was chemically synthesized with the reactive triple bond masked, and the click-chemistry addition between the azide and dibenzocyclooctyne was activated by UV exposure at 350 nm (Fig. 4). The labeling products were evaluated with fluorescence microscopy.

2-aryl-5-carboxytetrazole (ACT) has been used as a photo-reactive label for drug target identification.[37] ACT was covalently attached to drug candidates such that the modification did not significantly change their binding affinity and specificity. A reaction mechanism was proposed (Fig. 5) in which, after ligand binding, a carboxy-nitrile imine intermediate is generated following UV exposure at 302 nm, which then reacts with a nucleophile (e.g., the carboxylate group on Glu side chain) to form a covalent bond with the target protein near the active site. Binding site analysis was achieved with mass spectrometry (MS). The efficiency in capturing desired targets *in vivo* using the ACT label was comparable with the that of diazirine-labeled ligands in this study.

S-adenosyl-L-methionine (AdoMet) is a natural substrate for methyltransferases. The methyl group on the sulfur atom can be transferred by methyltransferase to biological targets in the cell.[38] With UV exposure, AdoMet itself was shown to specifically label several methyltransferases.[39,40] This method has been used to study the binding site of AdoMet.[41,42] An analog of AdoMet has also been used to specifically label DNA.[43]

Applications

1. Protein-protein interactions

Photo reactive reagents have been used to study protein-protein interactions *in vivo*. These reagents tend to have structures similar to those of natural amino acids and can be site-specifically incorporated into the protein. UV irradiation *in situ* induces the formation of covalent protein-protein adducts, allowing transient protein-protein interactions to be captured.

L-photo-leucine and L-photo-methionine are two diazirine compounds that can be chemically synthesized (see Fig. 7A) and are available commercially. Their structures are similar to the corresponding natural amino acids. Photo-leucine and photo-methionine have been successfully incorporated into membrane proteins through unmodified mammalian translation machinery of COS7 (monkey kidney) cells.[1] These two unnatural amino acids were nontoxic to these cells. Different protein-protein complexes linked with covalent bonds were detected by western blotting. Photolytic labeling enabled a new protein-protein interaction between membrane protein PGRMC1 and Insig-1 to be identified, which is involved in the regulation of cellular lipid homeostasis. In a study of Alzheimer's disease, photo-leucine and photo-methionine were used to confirm the preferential binding of soluble oligomeric assemblies of amyloid β peptides (ADDLs) with a subunit of AMPA receptors in neurons.[44] A similar method utilizing these two photo-reactive reagents was used to detect a direct interaction between subunits of RNA polymerase II and a mediator protein complex in eukaryotic cells.[45]

Several diazirine compounds with structures similar to lysine have been used to study protein-protein interactions. 3'-azibutyl-N-carbamoyl-lysine (AbK) has the diazirine functional group attached at the end of the lysine side chain. An approach using a combination of AbK photolytic labeling and stable isotope labeling of amino acids in cell culture (SILAC) allowed protein interactions with histone subunits to be identified in HEK293T cells.[46] A second photolytically labeled lysine that retains the primary amine on

the side chain was site-specifically incorporated into histone and used to identify histone and chromatin binding proteins during post-translational modifications in HeLa cells.[47] In order to expand the spatial range in which interactions could be detected, N^{ε}-[((4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)-benzyl)oxy)carbonyl]-L-lysine (TmdZLys) was prepared to detect possible protein interactions, with a spacer arm of 15 Å.[48] The structures of AbK, photo lysine and TmdZlys are shown in Fig. 7B.

The benzophenone compound p-benzoyl-L-phenylalanine (pBpA) has been used for protein-protein interaction studies *in vitro* and *in vivo* following procedures similar to those described above.[27] pBpA has also been used to study protein-DNA binding.[49] Another diazirine derivative of phenylalanine, 4'-[3-(trifluoromethyl)-3*H*-diazin-3-yl]-L-phenylalanine (TmfdPhe), has been genetically encoded in *Escherichia coli* and could be used in photolytic labeling *in vivo*[50], though results have not been reported. Structures of pBpA and TmfdPhe are shown in Fig. 7C.

2. Protein-ligand binding

Photolytic labeling can be used to identify the interactions involved in ligand binding. For ligands that interact with proteins non-covalently, it is often difficult to detect their target *in situ*. Ligands with photo-reactive functional groups can be activated after UV exposure to form covalent bonds with the target protein. The ligand-protein complex can then be analyzed with methods such as SDS-PAGE and liquid chromatography mass spectrometry (LC-MS). Known ligands with photo-reactive functional groups can also be used to discover off-target interactions, and to study selectivity in ligand-protein binding.

In early development, many drugs are designed to be ligands that are selective for specific targets. Off-target interactions are generally undesirable, as they can lead to unexpected side effects.[51] However, the study of off-target interactions may expand the understanding and effective usage of existing drugs, and may enable the discovery of new drugs or facilitate screening of drug candidates for a range of targets early in development.[52] Dasatinib is a chemotherapeutic agent that targets the Src/Abl family of tyrosine kinases, and is used for the treatment of imatinib-resistant chronic myelogenous leukemia (CML).[53] DA-2, a dasatinib analog with its core structure maintained, was used to establish a proteome profiling method to identify off-target interactions of dasatinib *in vivo*.[54] DA-2 differs from dasatinib in that a diazirine and an alkyne were introduced through chemical synthesis. DA-2 was internalized by both K562 and HepG2 cancer cells and bound to various intracellular targets. Following UV exposure, covalent bonds formed between DA-2 and the target proteins through the diazirine moiety. Two click reporters, rhodamine and biotin, each modified to contain an azide group, were then reacted with the DA-2 alkyne through click chemistry, allowing the ligand-complexes to be purified and analyzed. Several serine/threonine kinases were identified with this method, and their activities were validated by pull-down/immunoblotting experiments and kinase inhibition assays. In this study, the photolytic labeling method was able to identify more putative targets than an immobilized dasatinib affinity matrix. Similar diazirine and alkyne modifications have been made to the inhibitor MLN8237, which targets the ATP-binding site of kinases. The off-target interactions of MLN8237 were identified.[55]

Angiotensin II (AngII) is an octapeptide hormone that activates AT_1 and AT_2 receptors and functions in the regulation of the cardiovascular system.[56] Some truncated analogs of AngII have been found to be biological active, but their receptors are difficult to distinguish from one another. Two photo-reactive analogs of the hexapeptide AngIV, [N_3-Phe^6]AngIV and [Bpa^6]AngIV, were developed with the photo-reactive functional group at the phenylalanine residue.[57] [N_3-Phe^6]AngIV underwent the phenyl azide photo-reaction (Fig. 1) and [Bpa^6]AngIV underwent the benzophenone photo-reaction (Fig. 3). [N_3-Phe^6]AngIV and [Bpa^6]AngIV showed high affinity for the AT_4 receptor from bovine endothelial membrane. Subsequent analysis of AT_4 showed that this receptor is a 186 kDa integral membrane glycoprotein.

3. Structures of proteins and their oligomers

Photolytic labeling can be used to form covalent bonds between nearby residues within proteins themselves. Following UV exposure, analysis of the digested peptides provides information on protein structure. Three hetero-bifunctional probes that are both isotopically-labeled and photo-reactive have been developed to study protein structure.[5] All have an NHS ester in their structure that reacts preferentially with primary amines, allowing derivatization of lysine groups in the protein. The photo-reactive functional groups on the probes were phenyl azide (ABAS-$^{12}C_6/^{13}C_6$), diazirine (SDA-$^{12}C_5/^{13}C_5$) and benzophenone (CBS-$^{12}C_6/^{13}C_6$). Proteins were first incubated with the probe in the dark to allow reaction with the NHS ester, followed by UV exposure to induce covalent bond formation within ~ 5 or 7 A of the lysine nitrogen atom. ABAS and CBS were able to detect crosslinks in a well-established system of RNase S. The probe ABAS was also used to study the structure of a disordered protein, α-synuclein, which is implicated in neurodegenerative disease. Multiple interactions within the protein were identified.

Transient protein-interactions, such as oligomerization dependent on post-translational modifications, can be difficult to capture. To address this problem, photo methionine was site-specifically incorporated into the MH2 domain of the Smad2 signaling protein, which forms homo-trimers in response to the phosphorylation of serine residues.[58] This method was compatible with both solid-phase peptide synthesis and expressed protein ligation. The transient MH2-MH2 interaction was captured with photolytic labeling. In a study of $A\beta_{16\text{-}22}$, its photo-reactive analogs were generated and incubated to form aggregates.[59] Photolytic labeling of the diazirine analog within the aggregates was successfully used to characterize the inter-peptide interactions. The insertion sites were identified with LC-MS analysis.

4. Protein-matrix interactions in lyophilized solids

Lyophilization, also known as freeze-drying, is widely used to create dried powders of therapeutic proteins with the goal of preserving the protein and extending the shelf-life of the product.[60] Excipients such as disaccharides are usually included in the formulation, and are thought to contribute to stabilization by interacting with the protein in the solid matrix.[61] Understanding the interactions between the lyophilized matrix and the protein could contribute to rational formulation design and reduce the time-to-market for new protein drugs. At present, however, there is a lack of methods able to detect non-covalent protein-

matrix interactions in the solid state. Our group has shown that photolytic labeling reactions occur in solid powders and can provide information on the interactions between the protein and the lyophilized matrix.

In one approach to these studies, the photo-reactive reagent can be incorporated in the lyophilized matrix as an excipient. For example, photo leucine has been included in lyophilized apomyoglobin formulations at molar ratios of apomyoglobin to photo leucine from 1:20 to 1:100.[6] Sucrose was also included in the matrix. After lyophilization, the solid samples were exposed to UV at 365 nm to induce photo-reaction. The photolytic labeling products formed from apomyoglobin and photo leucine were analyzed at both intact and digested protein levels, and the sites of labeling were localized to specific peptide fragments. The distribution of the photo leucine label on apomyoglobin depended on the ratio of photo leucine to apomyglobin in the sample, and gave an indication of the local interactions between apomyoglobin and photo leucine in the lyophilized matrix.

In an alternative approach, the photo-reactive reagent can be site-specifically incorporated into the protein to study its interaction with surrounding environment in the lyophilized solids. Succinimidyl 4,4'-azipentanoate (SDA) was used to react with lysine residues in order to introduce a diazirine functional group into myoglobin.[22] The addition of the diazirine label did not disturb the overall structure of myoglobin. Diazirine-labeled myoglobin was then lyophilized with either raffinose or Gdn HCl to detect protein-excipient interactions in the solid state. After proteolytic digestion of the labeled products, peptide-peptide, peptide-water and peptide-excipient adducts were identified by LC-MS.

Future Directions and Challenges

It has been nearly 50 years since photolytic labeling was first introduced to study protein-protein interactions *in vivo*[3] Since that time, photo-reactive reagents have been mostly used in solution environments to study protein-related interactions. Successful application of the method to study protein-matrix interactions in solid samples has been reported only recently. [6] The photolytic labeling reagents that have been discussed in this mini-review are summarized in Table 1, together with their advantages and disadvantages, as well as applications that have been reported. This section describes potential future developments of photolytic labeling technologies in protein drug development, as well as challenges that may be associated with these developments.

As noted above, photo-reactive reagents can be introduced into protein structure site-specifically through cell translation machinery. Protein-protein complexes can then form *in situ* through photo-activated covalent bonds, which allow them to remain stable through common detection methods. An advantage of this approach is that the location of the photo-reactive functional group can be controlled. However, this approach is limited by the availability of photo-reactive amino acid analogs, and by the ability of the derivatized protein to be taken up by cells. Photo leucine and photo methionine are commonly used reagents and are preferred for their small size and minor change to the side chain structure of the corresponding amino acids. Other reagents such as pBpA have also been used, but their structures mimic those of natural amino acids less closely. Some photo-reactive reagents

simply cannot be recognized and utilized by cells if their structures differ too greatly from natural amino acids. Thus, there is a need for a broader range of photolytically labeled amino acid analogs that can be efficiently expressed in proteins of interest and recognized by cells.

The photo-reactive moiety can also be introduced into protein through a post-translational chemical reaction. As noted above, the bifunctional probe succinimidyl 4,4'-azipentanoate (SDA) has been used in a number of protein-protein interaction studies, and contains a diazirine group and an NHS ester. The process of introducing the diazirine functional group into the protein via the reaction of NHS with lysine is relatively simple, but changes the size and charge of the lysine side chain. If lysine plays a critical role in the interaction to be studied, the results can be misleading. Moreover, the diazirine label cannot be introduced site-specifically with this approach. In addition to its expected reaction with lysine, the NHS ester may also react with serine, tyrosine and threonine.[63] Due to the heterogeneity of labeling, a large number of photolytically labeled species may be produced that differ in the extent and sites of labeling. This can make it difficult to analyze the derivatized protein and the products of its photolytic reaction at either the whole protein or peptide level. Similarly, quantitation of photolytic labeling products is complicated by this heterogeneity. Thus, there is a need for site-specific chemical labeling approaches that can be applied post-translationally to produce uniformly labeled protein.

The selection of appropriate photo-reactive reagents for a given study is also a concern, since the use of different photo-reactive reagents has been shown to give different results in some cases. Benzophenone, diazirine and phenyl azide labeling were compared in a study to identify the target proteins of PPAR-e and Tubulin-i.[64] The investigators found that the target proteins that were identified depended on the type of photo-reactive reagent that was used. In a study of $A\beta_{16\text{-}22}$ amyloid structure, three analogs containing phenyl azide (PA), phenyl trifluoromethyldiazirine (TFMD) and benzophenone (BP) were generated. The TFMD analog generated only intermolecular peptide-peptide products and provided straightforward information on aggregate structure. The information from PA and BP was less clear and more difficult to interpret.[59] A better understanding of the mechanisms of photolytic labeling reactions in various media would help in interpreting the results. Most commercial photolytic labeling reagents undergo radical reaction. However, the mechanisms are not fully understood in specific reaction environments, including the solid state, and the effects of parameters such as temperature, pH, viscosity and crowding are unknown. Some photo-reactive reagents show preferences towards particular amino acids, as mentioned previously, which can bias the results if these preferences are not recognized. Knowing these preferences and the factors controlling photolytic reactions would enable better use of this approach and further expansion of its application. Similarly, very little is known about the toxicities of photolytic labeling reagents or their more subtle effects on cellular processes. While photolytic reagents that are expressed or introduced into cells are often assumed to be non-toxic if the cells remain viable, it is possible that the pathways of interest are affected by the non-native functional groups, even before photolytic activation. *In vitro* and *in vitro* results using photolytic labeling could be interpreted with greater confidence if these effects were better understood.

Photo-reactive functional groups can also be incorporated into other molecules that are relevant for the discovery and development of protein drugs. For example, in formulation development, excipients with photo-reactive functional groups could be incorporated into the formulation to study protein-excipient interactions. Following the photolytic reaction, the resulting protein-excipient complex could be analyzed with methods such as HPLC and LC-MS with high resolution. The results would shed light on the protein-excipient interactions associated with protein stability and could lead to a better understanding of protein-excipient interaction mechanisms, all of which would benefit formulation design in the future. To facilitate these studies, there is a need for photolytically labeled compounds that mimic the excipients commonly used in protein formulation, including sugars and stabilizing amino acids (e.g., histidine).

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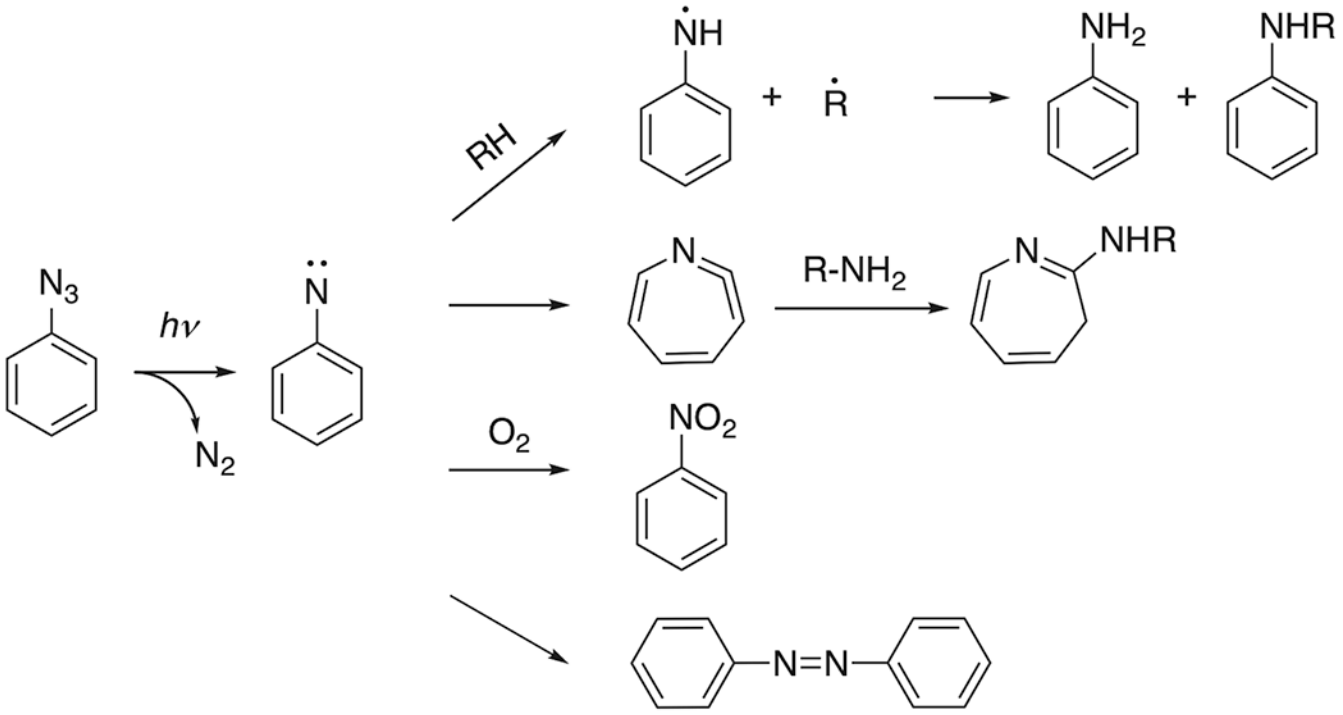

Fig. 1.
Reaction pathway of phenyl azide reagents. Adapted from [10] and [13].

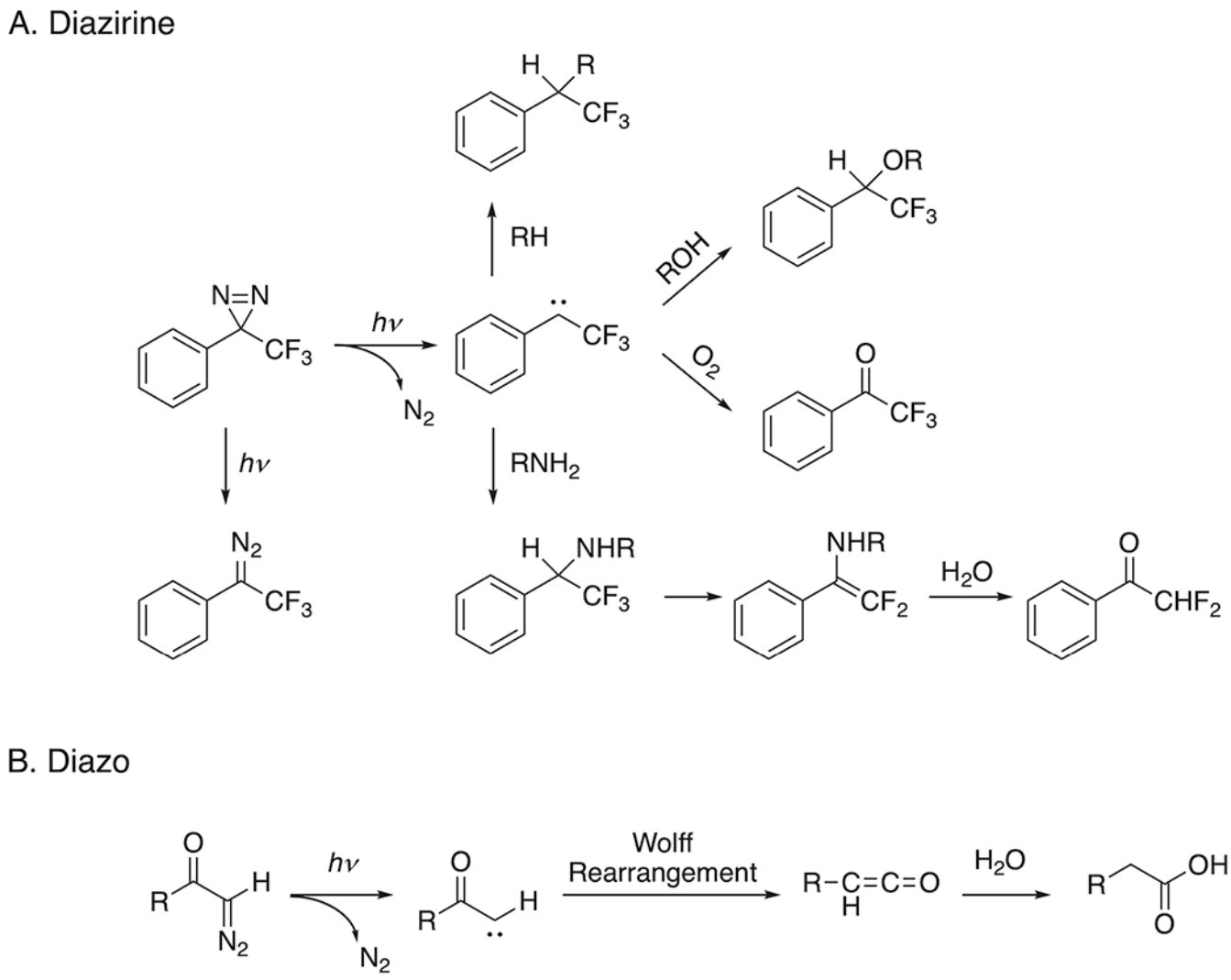

Fig. 2.

Reaction pathway of diazirine with trifluoromethylphenyldiazirine as an example (A) and diazo ketone showing Wolff rearrangement (B). Adapted from [16] and [19].

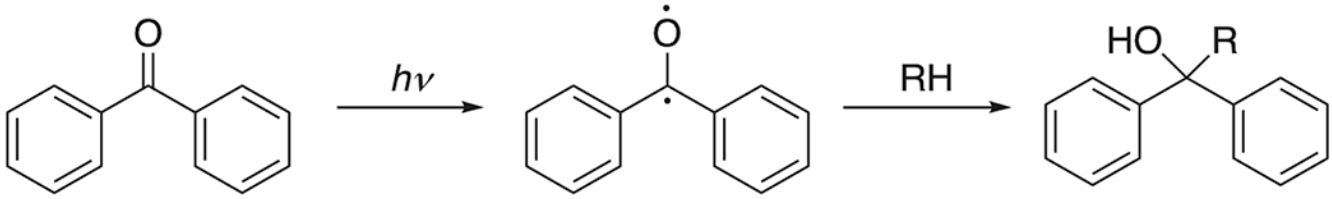

Fig. 3.
Reaction pathway of benzophenone. Adapted from [30].

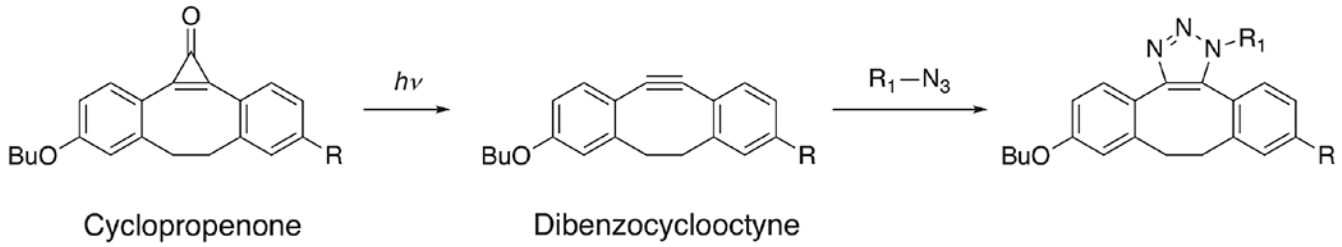

Fig. 4.
An example of photo-triggered click chemistry reaction in living cells. Adapted from [36].

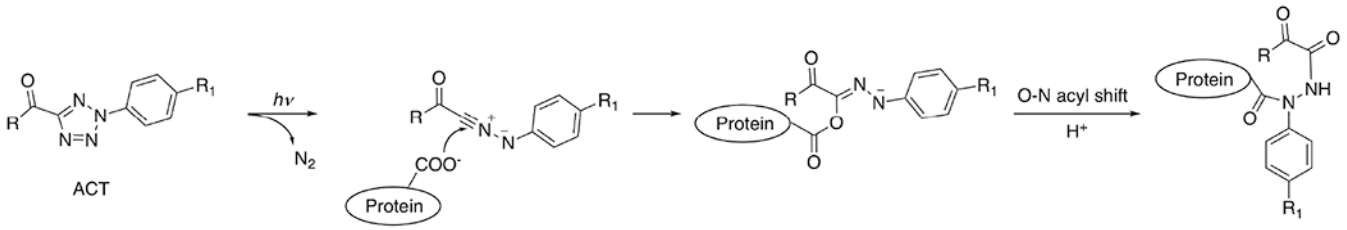

Fig. 5.
Proposed mechanism of ACT reaction. Adapted from [37].

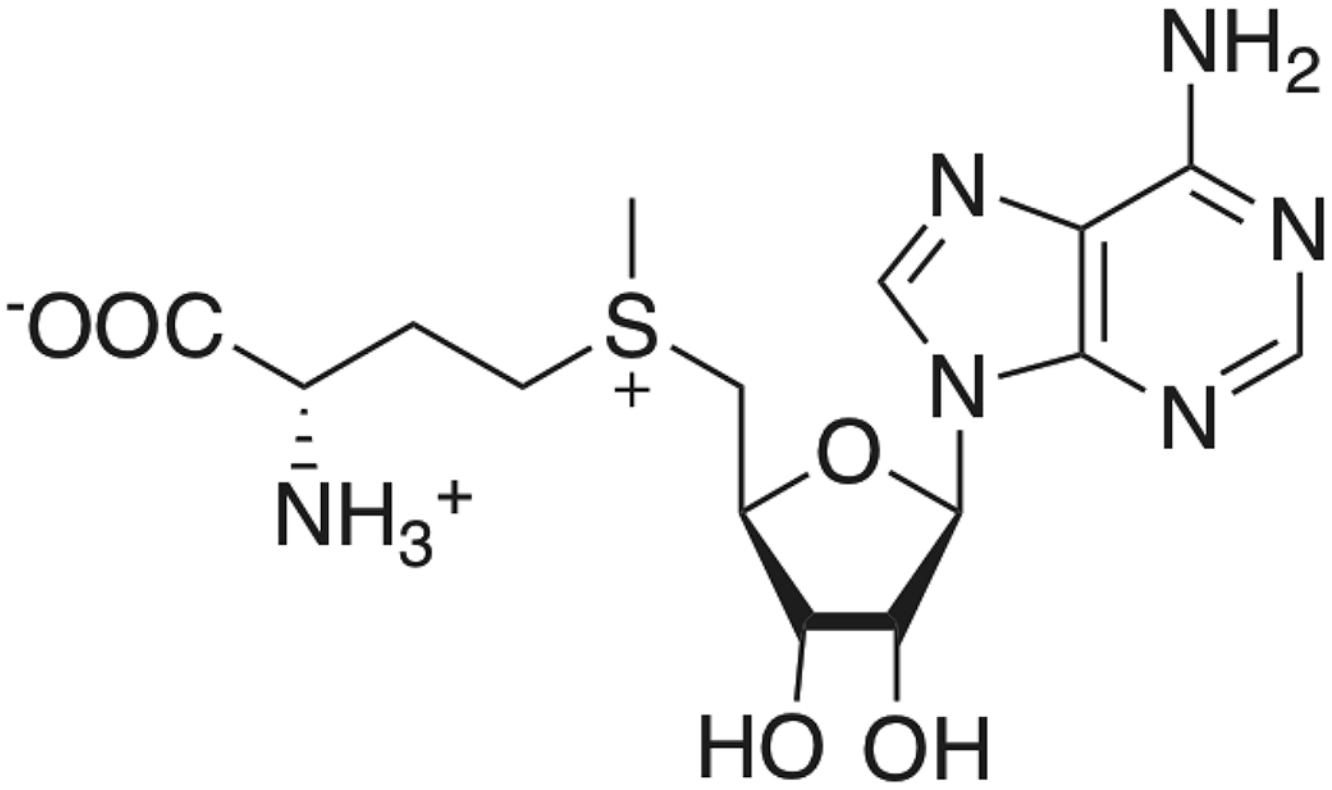

Fig. 6.
AdoMet structure. Adapted from [38].

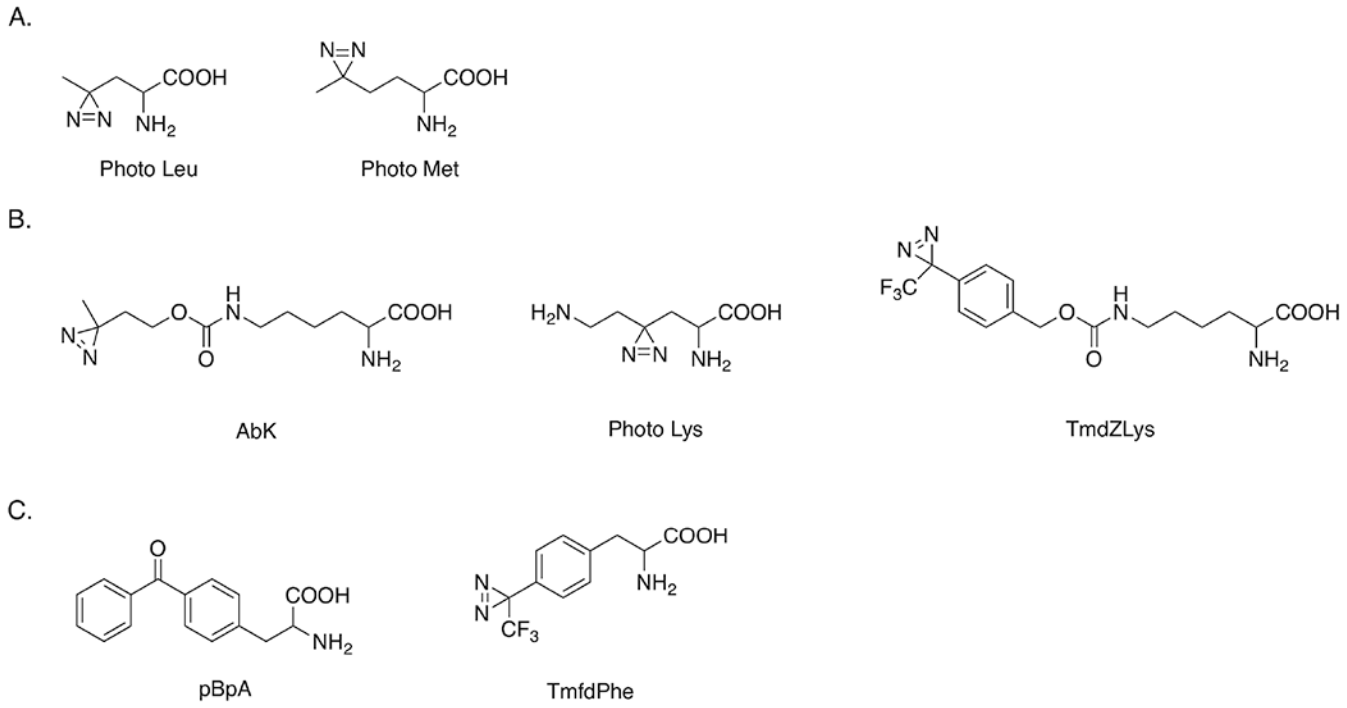

Fig. 7.

Structures of photo-reactive amino acids site specifically incorporated into proteins *in vivo*. Adapted from [1,27,46–48,50].

Table 1.

Photo-reactive reagents and their applications

Reagent[1]	Applications[2]	Advantages	Disadvantages	Refs
Phenyl Azides				
p-azido-L-phenylalanine	PPI	- Structural similarities to phenylalanine	- Short wavelength light required - Low labeling yield - Unexpected side products	7
ABAS-$^{12}C_6/^{13}C_6$	PS			5
TmfdPhe	NR			50
Carbenes				
Photo Leu	PPI, PMI	- Small size - High labeling efficiency - Activation wavelength safe for proteins	- Unexpected side products	1, 6, 44, 45
Photo Met	PPI			1, 44, 45
AbK	PPI			46
Photo Lys	PPI			47
TmdZLys	PPI			48
TFMD	PA			59
NHS-SDA	PS, PMI			5, 22
DA-2	PLB			54
Benzophenones				
pBpA	PPI, PLB, PDB	- Genetically encoded	- Limited structural flexibility	27–29, 49
CBS-$^{12}C_6/^{13}C_6$	PS			5
Other Reagents				
Cyclopropenone	Click	- Largely unknown	- Largely unknown	36
ACT	Target ID			37
AdoMet	Labeling			39–42

[1] See text for abbreviations and full chemical names.

[2] Abbreviations: PPI = protein-protein interactions, PS = protein structure determination, NR = none reported, PMI = protein-matrix interactions in solid samples, PA = protein aggregates, PLB = protein-ligand binding, PDB = protein-DNA binding, Click = photo-activated click chemistry, Target ID = drug target identification, Labeling = labeling of methyltransferases and DNA.