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DIAZIRINE-BASED MULTIFUNCTIONAL PHOTO-PROBES FOR AFFINITY-BASED ELUCIDATION OF PROTEIN-LIGAND INTERACTION

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Abstract – Diazirine-based photoaffinity labeling is recognized as one of the most reliable methods for analyzing protein-ligand interactions. Despite its excellent chemical and physical properties, the labeling process consists of an inefficient series of analytical steps: probe preparation, detection and isolation of the target protein, and identification of the labeled site within the active site. Firstly, this review introduces a versatile method for derivatization of trifluoromethylphenyldiazirine, the most common photocross-linker, then discusses its use in optimizing the probe for the simplification of labeled protein analyses including selective detection and purification, and the resulting acceleration of target protein identification by structural analysis. The present review reflects recent achievements in the chemistry and biological application of diazirine photoaffinity probes.

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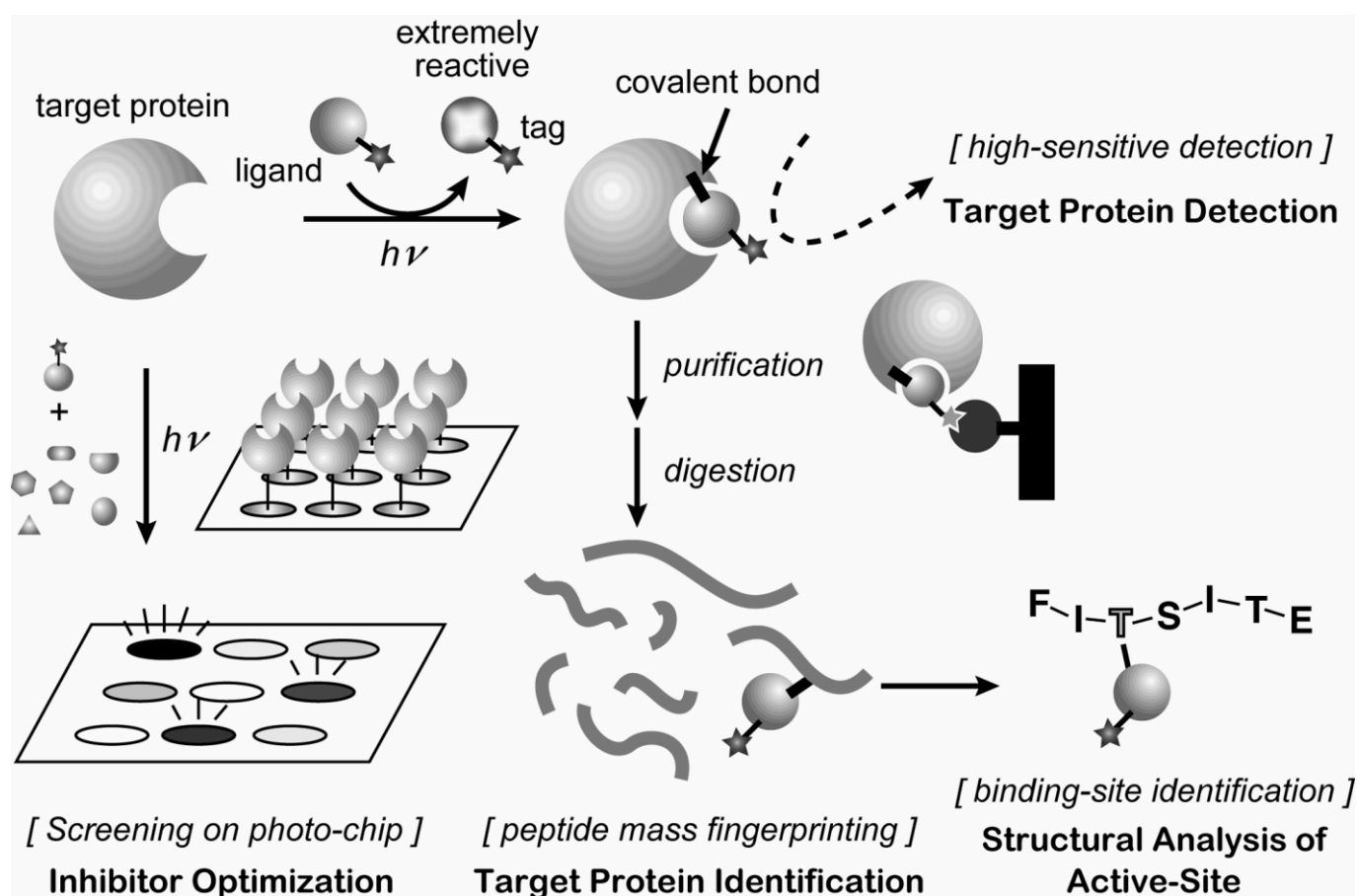
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1. CHEMICAL APPROACHES IN THE ANALYSIS OF BIOMOLECULAR INTERACTION

Ligand-protein and protein-protein interactions are fundamental for all cellular functions, and identification of the macroscopic and microscopic molecular interactions within these complexes is essential for understanding their expression mechanisms. Fluorescence-based imaging has remarkably contributed to the current understanding of the physiological functions of biomolecules through spatiotemporal visualization of the target molecule in a cell or tissue.¹ These techniques are used to analyze the association and dissociation of proteins at cellular level. Although the structure-based analysis of the interacting surface is important for elucidating protein functions, few analytical methods respond quickly enough to deal with the micro- to nano-mole of trace samples. The crystallography is capable of describing interactions even in protein complexes through three-dimensional structural analysis at atomic level, but the crystallization is late determining step and the throughput of methodology is not enough to deal with an increasing number of purified proteins. Recent techniques in high-resolution NMR analysis, such as saturation transfer difference and diffusion ordered NMR spectroscopy,² have allowed the elucidation of ligand and protein binding state applying large sample sizes and time-consuming measurements. Covalent cross-linking of interacting molecules is an alternative method that allows profiling of interacting proteins that are difficult to purify and also provides the structural data of interacting sequences within proteins. In particular, cross-linking allows noncovalent interactions, including transient and/or weak binding, to be captured as stable covalent complexes that maintain the information of contact points during subsequent purification, biochemical treatments, and analysis. Additionally, this method is useful for collecting the information of higher heterogeneous complexes such as membrane systems and self-assembling/dispersing proteins. This review will describe the recent developments of affinity-based cross-linkers, tags, and the application of these probes to obtain desirable information at the molecular, domain, and atomic interaction level.

2. PHOTOAFFINITY LABELING

Affinity-based labeling methods are commonly employed to determine interacting partners and interaction sites, which is composed of specific cross-linking, purification of labeled protein, protein digestion, isolation of labeled fragment, and mass analysis. The labeled protein can be selectively monitored via sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using fluorescence or radioisotopes, and identified by peptide mass fingerprinting (PMF) analysis, a mass pattern analysis of peptide fragments compared with *in silico* digests, after purification. The interaction site can be identified by amino acid sequencing of the labeled peptide by tandem mass spectrometry (MS). The important features required for the identification of the target are the reliability of cross-linking, ease of preparation and handling of the probe, efficient purification of labeled products, and clarity of analytical data. To increase the quality of information provided from cross-linking reactions, cross-linker and tag choices are important as well as the specificity of biological probe to its interacting biomolecule.



Scheme 1. Target profiling using photoaffinity labeling

Photoaffinity labeling (PAL) has been used to identify and localize binding proteins and map their interacting regions since the early 1960s (Scheme 1).³ In PAL, a covalent linkage is created between a photoactivatable ligand and a macromolecule upon irradiation in a reversibly bound state. Specific photo-induced cross-linking methodologies have been developed with consideration for the

chemistry/photochemistry of the photophore and the chemistry of the reactive intermediate.⁴ Ideal photoreactive groups are stable under physiological conditions, small to minimize steric perturbations, and capable of generating a short-lived, promiscuous, highly reactive species by light irradiation after reaching the target molecule. Thus, the active species can instantaneously form a covalent bond with molecules within a certain volume with minimum diffusion. Unlike conventional chemical labeling, identification of nonspecific interactions is greatly reduced, which gives high reliability through target-specific labeling. After the specific photoinitiated covalent coupling reaction, the target protein may undergo irreversible activation or inactivation, and concurrently, the functional site is labeled with a detectable tag, which allows easy determination of its location. Despite these advantages, the yield of these reactions is generally low, which is a critical problem for in situ detection of low-abundance proteins. Therefore, PAL has been generally employed for target identification and not molecular imaging.

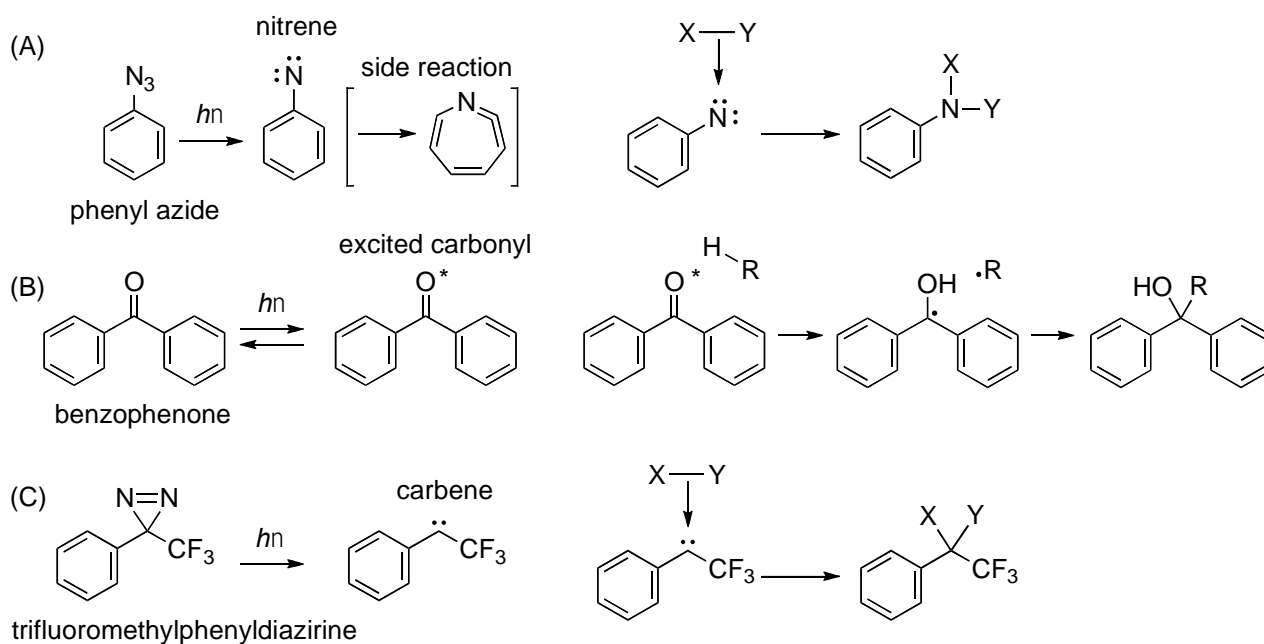
3. DIAZIRINE PHOTOPHORES FOR PAL-BASED PROTEIN CAPTURE

3-1. Advantages of Diazirine over Other Photophores

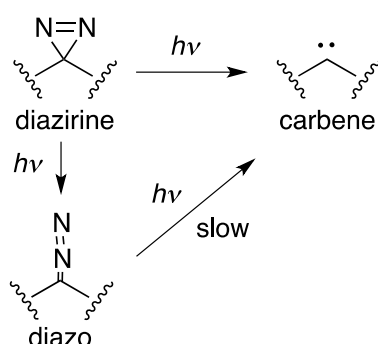
In general, aryl azide, benzophenone, and diazirine are often used as photophores. The photochemistry of these groups has been extensively investigated for use in biological photolabeling.⁵ Aryl azide is activated with ultraviolet (UV) light (<300 nm) and undergoes denitrogenation to generate a singlet nitrene that promptly penetrates the chemical bond of a proximal molecule (Scheme 2A). Aryl nitrene also undergoes two side reactions: a ring expansion reaction that rearranges to a ketenimine intermediate and hydrogen abstraction from the triplet state to produce a radical species. The ring expansion reaction can be suppressed by using a fluorinated aryl azide derivative. Azide has the advantages of small size that does not affect ligand affinity and easy employment through its insertion into aromatic rings of a variety of ligand molecules. These properties are important for labeling the interacting site. However, aryl azides are relatively unstable under reducing conditions, including in the presence of a thiol compound, and the cross-linked products can be labile when the nitrene forms a bond with a heteroatom.

Benzophenone is excited by long-wave UV irradiation (~360 nm) and abstracts a hydrogen from an adjacent molecule to generate a biradical, which then forms a covalent bond (Scheme 2B). Unlike the nitrene or carbene, the excited carbonyl group does not react with water and relaxes back to the ground state. Therefore, cross-linking occurs more slowly, but the labeled product increases as a function of irradiation time to give much higher yields than diazirine and aryl azides, which generally give only a few percent yield. In spite of its large, hydrophobic structure, synthesis of benzophenone derivatives is easy and has supplied a variety of modified ligand probes so far, including lipid analogs for membrane protein

analysis in hydrophobic environments.⁶ However, it should be noted that the photoreaction is selective with respect to its reaction partner. When used to identify the ligand-binding site within a protein, the label should be located at the interacting point. Notably, benzophenone preferentially cross-links to methionines.⁷ Therefore, misinterpretations of the cross-linking data occur frequently in binding site analysis.



Scheme 2. Photocross-linking mechanisms of common photophores; A) phenyl azide, B) benzophenone, and C) trifluoromethylphenyldiazirine



Scheme 3. Two pathways in diazirine photolysis

Diazirines are categorized as either aliphatic or aromatic based on the attachment of the diazirine ring directly to aliphatic carbons or to an aromatic ring, respectively. Under long-wave UV irradiation (~ 360 nm), diazirine produces a carbene intermediate by denitrogenation, and the carbene spontaneously forms a covalent bond with a proximal molecule through insertions, such as C-H, O-H, N-H, and C-C, which are mostly stable under physiological conditions (Scheme 2C). Brunner et al. developed an effective

aromatic diazirine for PAL in 1980 by introducing a trifluoromethyl group.⁸ Phenyl and trifluoromethyl substituents at the third position of the diazirine ring largely increase its thermal stability under general synthetic conditions and also prevent side reactions such as Wolff rearrangement and electrophilic substitution via diazo intermediates. Along with the production of the carbene species, diazirines partially isomerize to diazo intermediates (Scheme 3). Although diazo compounds undergo photolysis to yield a carbene,⁹ long-lived diazo compounds react with diverse nucleophiles as strong alkylating agents in the dark to induce unfavorable, nonspecific labeling that complicates the interpretation of the analytical data. Substitution at the third position of diazirine decreases the ratio of diazo formation, and the trifluoromethyl group reduces the reactivity of the diazo intermediate as an electrophile. Recently, PAL experiments using cinnamate¹⁰ and coumarin¹¹ derivatives were reported, and these corresponding diazo compounds were not detected upon photolysis by ¹H and ¹⁹F NMR studies. Since 3-trifluoromethyl-3-phenyldiazirine is rather bulky compared to azide, it might not be suitable for clarification of the interacting site but would be for identification of the peptides comprising the binding domain. Aliphatic diazirines have gained popularity in biological applications in the recent past because of their smaller size. The smaller alkyl diazirine derivatives¹²⁻¹⁴ are useful for close labeling and identification at the site of interaction, but these compounds are less stable (explosive) and easily produce long-lived diazo intermediates and rearrangement products. Recently, several synthetic methods for synthesizing diazirine rings have been reported via conversion from carbonyl group,¹⁵ which has allowed their direct incorporation into biomolecular structural components such as amino acids,¹⁶ sugars,¹⁷ lipids,¹⁸ and steroids¹⁸ (Figure 1, see Section 3-3). Since the rearrangement products should not be reactive, PAL using aliphatic diazirines can be suitable for the identification of the exact position of ligand interaction, while aromatic diazirines can be beneficial for protein/domain identification.

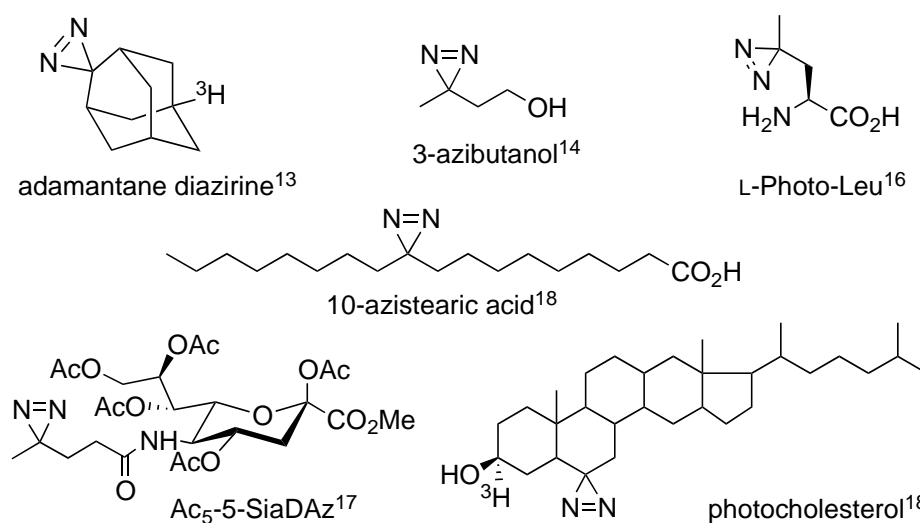
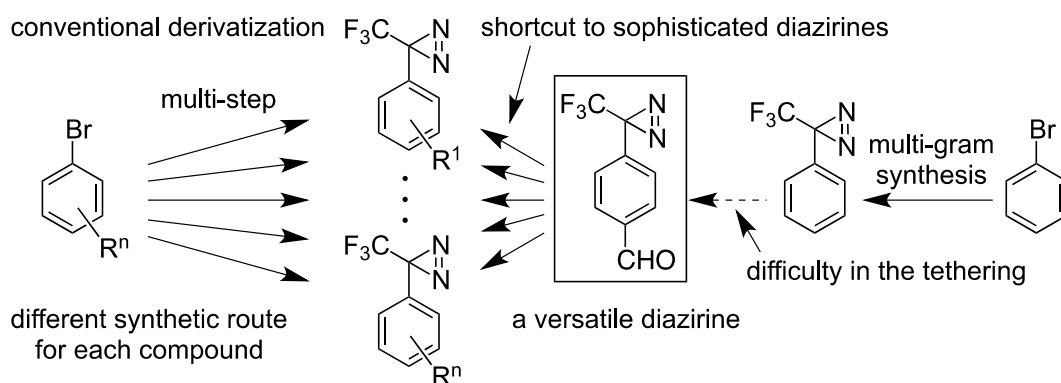


Figure 1. Examples of aliphatic diazirines and bioprobes for photoaffinity labeling

3-2. Versatile Synthetic Intermediates Leading to a Library of Various Compounds



Scheme 4. Synthesis of various aromatic diazirines via a versatile diazirine

Despite the useful properties of 3-trifluoromethyl-3-phenyldiazirine for target identification, the limited availability of conveniently modified derivatives compared to aryl azide and benzophenone has prevented widespread biological use. Most diazirine-attached photo-probes required preparation through repetition of time-consuming multistep syntheses from the initial materials in response to the ligand of interest (Scheme 4).¹⁹ Although large amounts of 3-trifluoromethyl-3-phenyldiazirine can be easily prepared for use as a building block, use of this compound has been hampered by the low reactivity of its benzene ring and the instability of the diazirine ring under the high temperature and acidic conditions of conventional Friedel-Crafts reactions.²⁰ Some trials have investigated the formation of C-C bonds on the benzene ring. Thallium-mediated direct carboxylation of compound **1** (Figure 2) gave only moderate yields of an isomeric mixture.²¹ Meanwhile, a Friedel-Crafts reaction with GaCl₃ and dichloromethyl methyl ether was found to produce the desired aldehyde derivative (**2**) in the presence of trifluoroacetic acid (TFA).¹⁹ This suggested that the diazirine group was stable in acidic medium, but large amounts of unreacted compound **1** were recovered. Using trifluoromethanesulfonic acid (TfOH), where dichloromethyl methyl ether was reported to be stable,²² Friedel-Crafts reaction successfully proceeded on inert benzene rings. Triflates of Ag, Pr, Y, Yb, Gd, and Sm were ineffective, whereas GaCl₃ and FeCl₃ afforded compound **2** in moderate yields (31% and 47%, respectively). Finally, both TiCl₄ and SbF₅ efficiently gave compound **2** in 80% yield in TfOH at 0 °C (Figure 2, table inset). Since direct formylation of an inactive benzene ring has been accomplished by using dichloromethyl methyl ether in TfOH, a variety of diazirines have been easily synthesized by converting the formyl group.²³ The aldehyde (**2**), as a key synthetic intermediate, was oxidized to the corresponding carboxylic acid (**3**) or ester (**4**) or reduced to a benzyl alcohol derivative (**5**) with sodium borohydride. Succinimide ester (**6**), an active ester, is useful for selective coupling with amino groups to make various photoactivatable bioactive molecules. Compound **5** was converted to a benzyl bromide derivative (**7**) by the Appel reaction as another key synthetic intermediate.

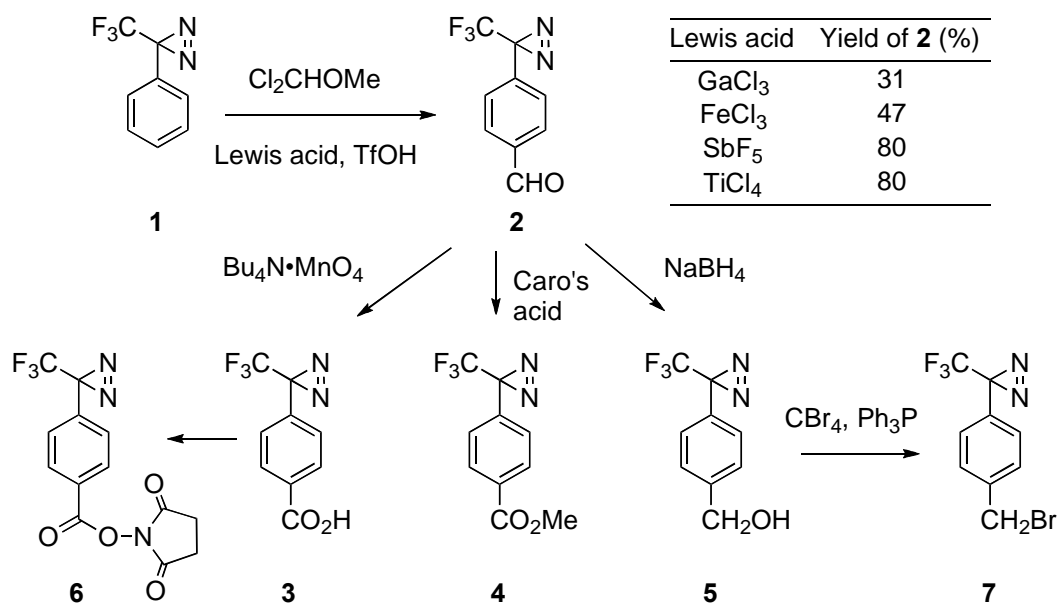


Figure 2. Synthesis of a key aromatic diazirine (**2**) and its derivatives

3-3. Probes for Biomolecules

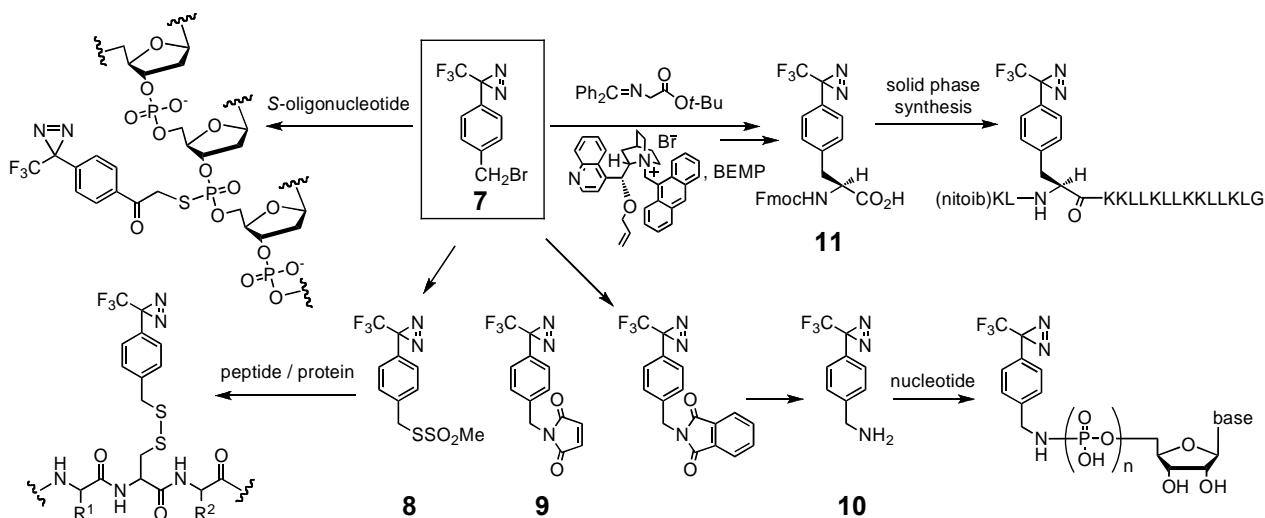


Figure 3. Preparation of photoactivatable bio-probes from a benzyl bromide analog (**7**)

The benzyl bromide derivative (**7**; Figure 3) is another key synthetic intermediate that can be converted to a corresponding thiosulfonate (**8**)²⁴ or maleimide (**9**),²⁵ for example. These derivatives, including bromide (**7**), can be used as SH group-selective reagents for probing cysteine residues in peptides/proteins to yield photoactivatable peptides/proteins. Photoactivatable oligonucleotides were also prepared from compound **7** by coupling with a commercially available phosphorothioate group, or prepared by phosphoramidate formation at the terminal phosphate using an amine derivative (**10**). An amine derivative (**10**) was prepared by Gabriel synthesis and coupled to a carboxylate or phosphate group for use as a nucleotide analog.²⁶ A phenylalanine (or tyrosine) analog (TmdPhe, **11**) was successfully prepared according to

Corey's asymmetric synthetic method²⁷ with excellent yield using a cinchonidine-based catalyst for the alkylation of *tert*-butylglycinate benzophenone imine with bromide (**7**), which was directly incorporated into a peptide at the desired position by conventional solid-phase peptide synthesis to yield various photopeptides.^{23,28} For example, PAL performed in HEK293T cells using the TmdPhe-incorporated photoactivatable analog successfully identified lanthionine synthetase component C-like protein 1 as a potential intracellular target of octaarginine, a representative cell-penetrating peptide.²⁹

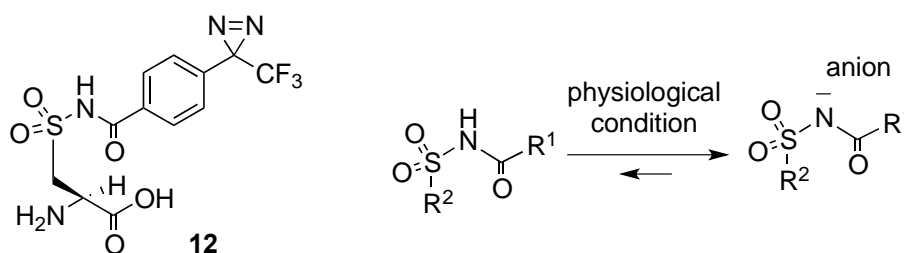


Figure 4. Photoactivatable acidic amino acid analog utilizing an *N*-acylsulfonamide linkage

Novel photoreactive analogs (**12**) were synthesized to mimic acidic α -amino acids by incorporating a cysteine derivative with an *N*-acylsulfonamide moiety within the side-chain structure (Figure 4).^{30,31} *N*-Acylsulfonamide is known as a carboxylic acid bioisostere in medicinal chemistry,³² and the pK_a value is close to that of a general carboxylic acid, which results in the presentation of a negative charge by deprotonation under physiological conditions. It has been broadly used with diazirine analogs and has specifically identified enzymes with specificity for α -amino acids, including glutamyl endopeptidase from *Staphylococcus aureus* V8 strain (V8 protease), L-glutamate dehydrogenase (GDH), glutamic oxalacetic transaminase (GOT), and L-glutamine synthetase (GSyn). These results indicated that various functions could be introduced on an acidic terminus via acylation while maintaining an anionic feature.

Protein probing has been generally achieved by chemoselective reactions with surface functional groups. However, it requires protein purification and a process for reactant removal. In this method, photocross-linkers are randomly attached to the protein, which may influence protein-protein interactions. Several approaches have been developed for site-specific insertion of unnatural amino acids, including the use of native chemical ligation, intein-based methods, and in vitro and in vivo protein translation systems that make use of chemically aminoacylated tRNAs. The recent advances in unnatural amino acid mutagenesis have made possible the site-specific incorporation of a wide variety of amino acids into proteins in living cells,³³ including the photoactivatable amino acids *p*Bpa,³⁴ *p*Azpa,³⁵ TmdPhe,³⁶ L-Photo-Trp,³⁷ L-Photo-Met,¹⁶ L-Photo-Leu,¹⁶ L-Photo-Ile,¹⁶ and L-Photo-Pro³⁸ (Figure 5), which yielded cross-linking at nearly zero distance without significantly disturbing the interaction.

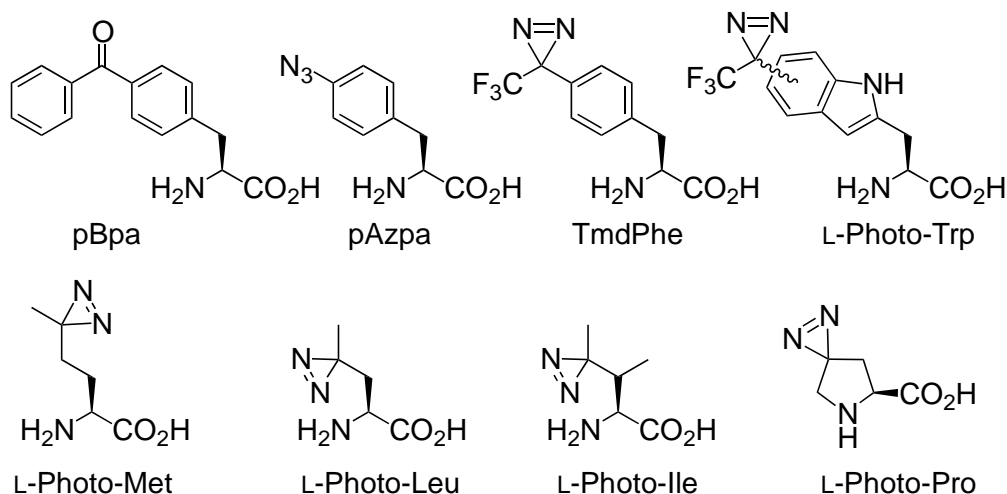


Figure 5. Photoactivatable amino acid analogs

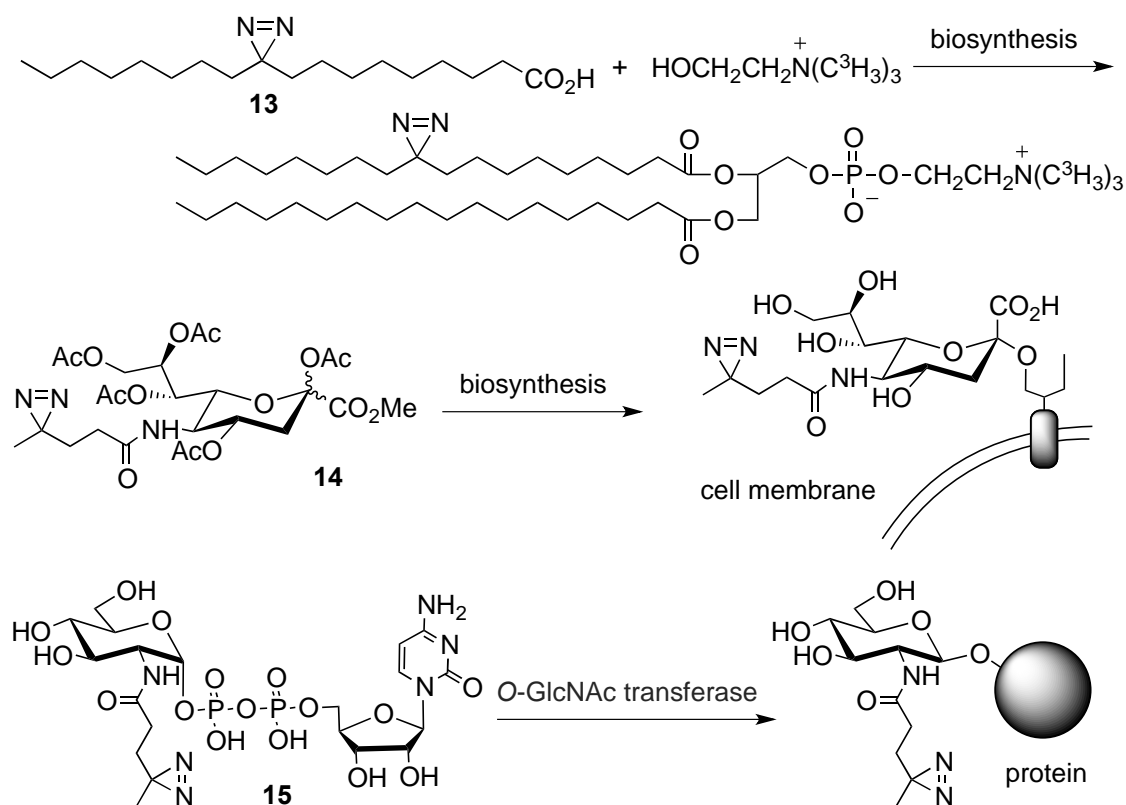


Figure 6. Biosynthetic incorporation of photoactivatable phosphatidylcholine and sugar

In addition to genetic incorporation, some photoactivatable amino acids, lipids, and sugars were biosynthetically or metabolically incorporated into biomolecules. Thiele et al. reported in situ biosynthetic incorporation of photoactivatable amino acids into an overexpressed protein in transfected mammalian fibroblast-like COS7 cells by cultivation in the presence of L-Photo-Met and L-Photo-Leu. Incorporation was nontoxic to cells, and the expressed photoactivatable protein successfully cross-linked to the interacting protein by irradiation to identify a regulator of cholesterol homeostasis.¹⁸ In a similar

way, photoactivatable phosphatidylcholine was generated in vivo from photoactivatable stearic acid (10-azistearic acid, **13**; Figure 6) and radioactive choline to investigate lipid-protein interactions.³⁹ Photoactivatable sugars (Ac₅-5-SiaDAz, **14**)¹⁷ were also metabolically incorporated into oligosaccharide chains expressed on the surfaces of living cells and competed efficiently with endogenous carbohydrate biosynthesis of sialic acid. In addition, the nucleotide-sugar UDP-GlcNDAz (**15**)⁴⁰ acted as a donor for O-GlcNAc transferase and was transferred to substrate proteins using intracellular posttranslational O-glycosylation. The photoactivatable proteins selectively cross-linked to their binding partners upon UV irradiation.

4. DETECTION AND PURIFICATION OF CAPTURED PROTEINS

4-1. General Features of PAL-Based Target Identification

The combination of PAL and SDS-PAGE is excellent in that it allows simultaneous proteomic analysis of multiple binding proteins with high resolution.⁴¹ To study DNA-protein binding, for example, the gel electrophoretic mobility shift assay (EMSA) is one of the most common methodologies. However, it is usually focused on investigating a particular protein of interest, and, although it can be performed in a limited condition of low salt concentration at low temperature, it cannot usually detect ternary complexes because of dynamic and reversible binding to DNA and weak-binding proteins. PAL by irreversible cross-linking to the interacting system can reveal EMSA-inaccessible proteins. These proteins can be selectively monitored using a tag such as a radioisotope, and binding specificity can be investigated in the presence of inhibitors by monitoring the relative emission strength of individual labeled proteins.

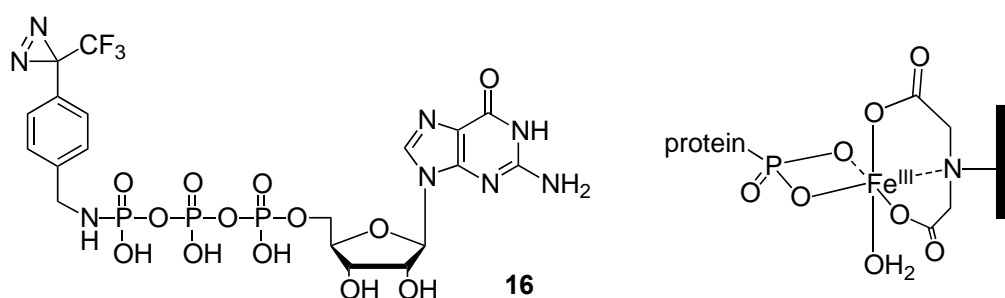


Figure 7. ATP photo-probe modified at the γ -phosphate and Fe³⁺-IMAC purification of an ATP-cross-linked product

In the PAL-based identification of target proteins, the low photocross-linking yield makes the process cumbersome and complicated, especially for analyzing low levels of target protein expression. Owing to the infinitesimal quantity of labeled product, the analysis usually proceeds by utilizing highly sensitive analytical tags such as fluorophores or radioisotopes or specific tags for selective isolation/enrichment, such as a biotin, perfluoroalkanes, and clickable groups. In some special cases such as nucleotide-labeled

products (16;²⁵ Figure 7), Fe³⁺-immobilized metal affinity chromatography (IMAC) can be applied for selective isolation targeting the phosphate backbone without incorporating an additional tag (Figure 7).⁴² Recently, more than 1000 phosphopeptides were efficiently enriched and identified by MS directly from HeLa cell lysate by using lactic acid-modified titania and β -hydroxypropanoic acid-modified zirconia metal oxide chromatography.⁴³

4-2. Biotin Tag

Radioisotopes such as ³H, ¹⁴C, and ¹²⁵I have been used from the early stages of PAL-based protein identification through introduction in the ligand molecule. They remain the most sensitive and widely used tags for quantitative analysis and retain ligand specificity. Radioactivity allows SDS-PAGE detection of the labeled protein and HPLC detection of the labeled peptide after digestion. Although the use of radioactive tags has greatly contributed to PAL studies for many years, radioactive exposure is a great health and environmental concern, and its use is institutionally regulated. To avoid radioactive contamination, a biotin tag is often installed on bioactive probes for chemiluminescence detection as a highly sensitive method. Due to the extraordinary strong biotin-avidin interaction ($K_d \approx 10^{-15}$ M), a biotinylated target protein of low concentration can be detected by chemiluminescence with a streptavidin-horseradish peroxidase (HRP) conjugate after blotting onto a membrane. Biotin is also useful for the purification of labeled proteins from the whole proteome as a highly selective sorting tag using an avidin-modified solid support. The strong binding interaction allows stringent washing conditions to efficiently remove proteins that are nonspecifically adsorbed on the surface.

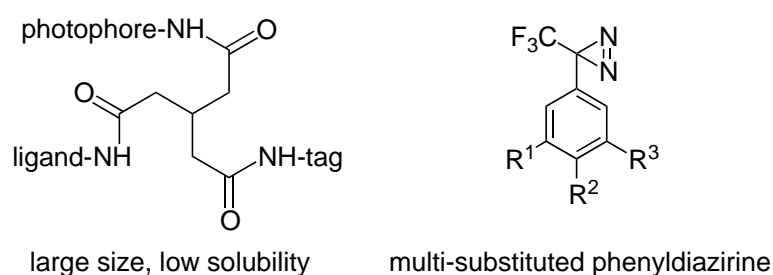


Figure 8. Structural units for multiple functionalization

Probe functionalization often involved a three-directional linkage with connections to a photophore (diazirine), ligand of interest, and tag (biotin), respectively (Figure 8).⁴⁴ However, this significantly increased the size of the reaction unit, which might interfere with the interaction, and the solubility was often significantly decreased because of the multiple amide bonds and biotin. Therefore, a trifluoromethylphenyldiazirine derivative equipped with a biotin into a benzene ring was newly synthesized as an all-in-one multifunctional reaction unit that maintained the solubility (Figure 9).⁴⁵

Recently, other modifications on diazirine rings were developed by replacement of the trifluoromethyl group with substituents such as a perfluoroalkyl chain for specific purification (see Section 4-4) or fluoromethyl connected with an azido or ethynyl group (see Section 4-6).

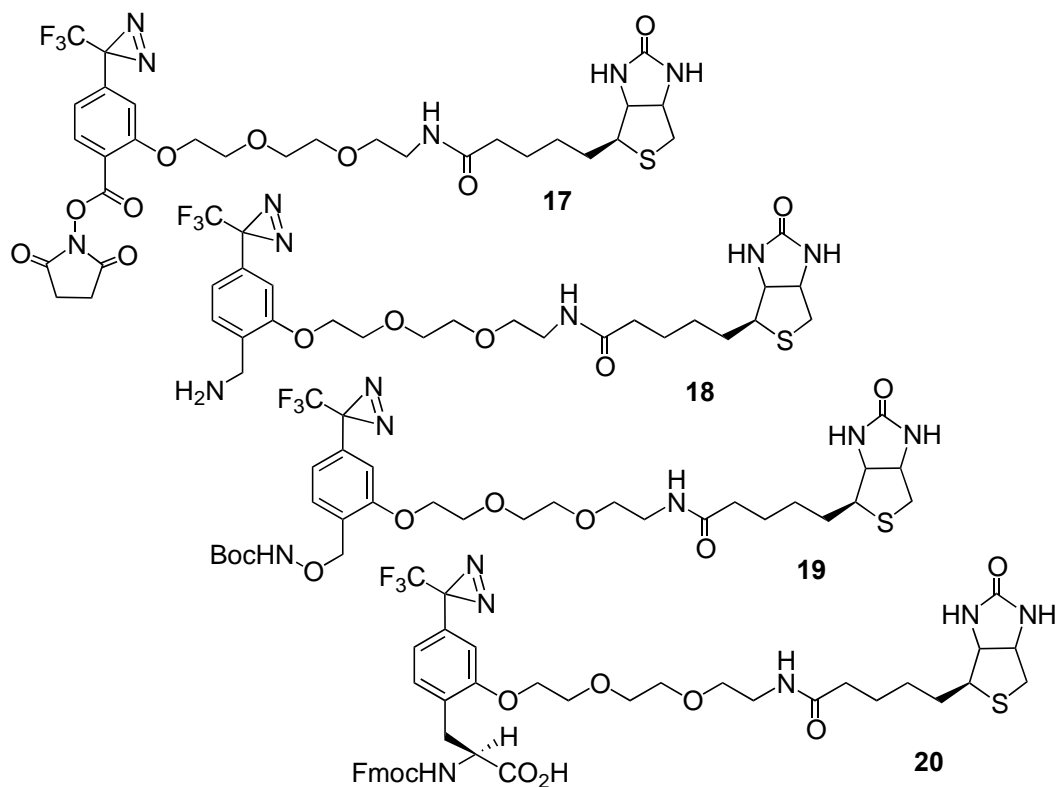


Figure 9. Biotinylated phenyldiazirine cross-linkers

A biotin group was connected through a polyethylene glycol-type spacer to a benzene ring at the ortho position for efficient capture of labeled protein using the avidin-immobilized support. Some useful derivatives were synthesized by substituting a functional group such as CO₂Suc (**17**; Figure 9),⁴⁶ CH₂NH₂ (**18**),²⁶ and CH₂ONH₂ (**19**)⁴⁷ for chemoselective modification of amino, carboxyl, and carbonyl groups, respectively, or an amino acid analog (**20**).²⁶ These were successfully applied to many biomolecules, especially for probing oligosaccharides. Compound **19** was easily and efficiently incorporated into oligosaccharides via an oxime ligation reaction of the aminoxy group and the aldehyde at the reducing end of the sugar chains through incubation at 37 °C and pH 5–6 without any protection (Figure 10). This procedure can be extended to the preparation of a variety of photoactivatable sugar analogs such as a sialyl Lewis X tetrasaccharide (**21**).⁴⁷ PAL studies with the photo-probes of oligosaccharides enzymatically released from sophoragrin (**22**; Figure 10) suggested that sophoragrin can sequester endogenous glycoprotein ligands via sugar-specific interactions,⁴⁸ and studies using photoactivatable chitobiose (**23**) revealed that the Na⁺/K⁺-ATPase β1 subunit is a potassium-dependent lectin that binds β-GlcNAc-terminating glycans.⁴⁹ The aminoxy derivative (**19**; Figure 9) was also used for direct

preparation of steroid hormones, the brassinosteroids (**24**; Figure 10), which helped to define a new steroid-binding domain of plant receptor kinase BRI1.⁵⁰

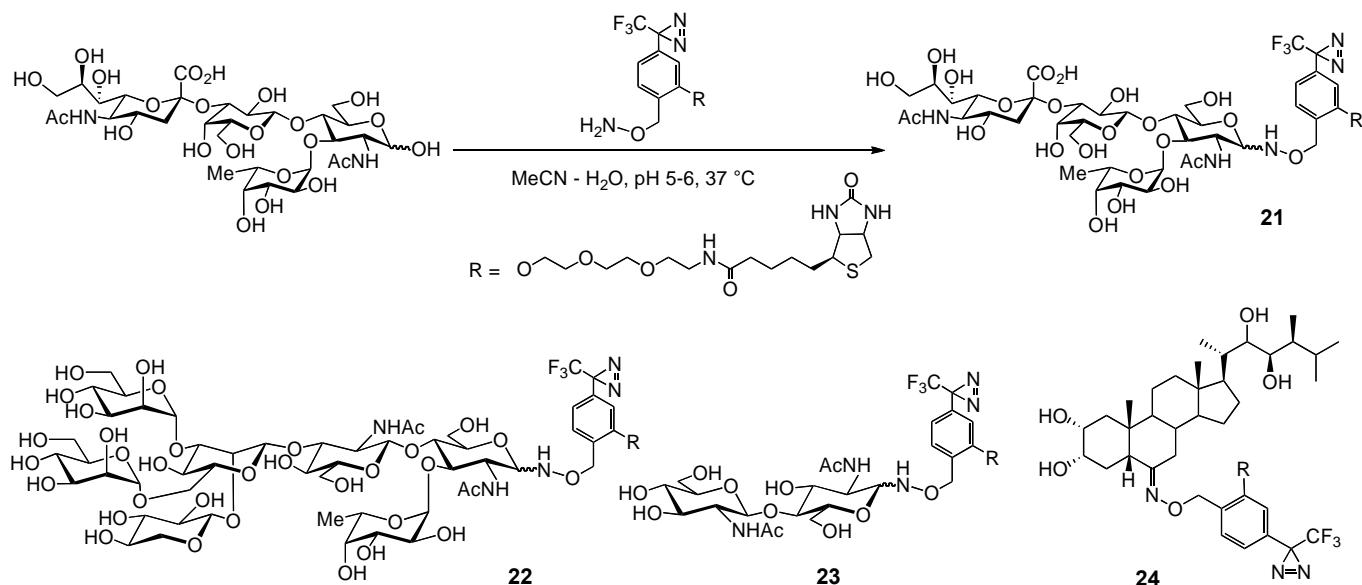


Figure 10. One-step preparation of photoactivatable saccharides and a steroid hormone

As proteins captured with these probes are already biotinylated, further chemical treatments should not be necessary for the detection and isolation of the labeled proteins. This invaluable feature accelerates identification of the target proteins, and the PAL identification process has been amazingly simplified and reduced to a few month's period. In the PAL of β -1,4-galactosyltransferase with a biotinylated *N*-acetylglucosamine analog (**25**; Figure 11),⁵¹ the labeled peptide fragment was enriched from an enormous amount of unlabeled proteolytic fragments by the specific interaction between biotin and avidin immobilized on the support. Soon after the PAL experiment, the structure of a complex consisting of the transferase and UDP-gal as the donor substrate was revealed by crystallographic analysis.⁵² PAL successfully identified the acceptor site of the enzyme, which was not determined by the crystallographic study.⁵³

A photoactivatable palmitoyl analog (**26**; Figure 11) was prepared from an amino acid derivative (**20**; Figure 9) and specifically captured multifunctional enzyme type 2 (MFE2), one of the peroxisomal β -oxidation enzymes, from rat liver peroxisomes.⁵⁴ Residues Trp249-Glu250-Arg251 were specifically labeled, where Trp249 is a critical residue for the dimerization of MFE2 and the starting point of a hydrophobic cavity leading to a catalytic residue (Tyr164). This was suggested by further PAL studies using MFE2 mutants obtained through replacing some residues in the cavity with hydrophilic ones, in which labeling efficiency was greatly reduced. The probe was designed as an analog the β -carbon for

oxidation with an amide moiety, which inhibited the enzymatic reaction but yielded efficient labeling. Cross-linker **20** worked as a branching unit in the synthesis of multifunctional probes; both amino and carboxyl functional groups were included for derivatization, and a biotin was added for target enrichment and detection.

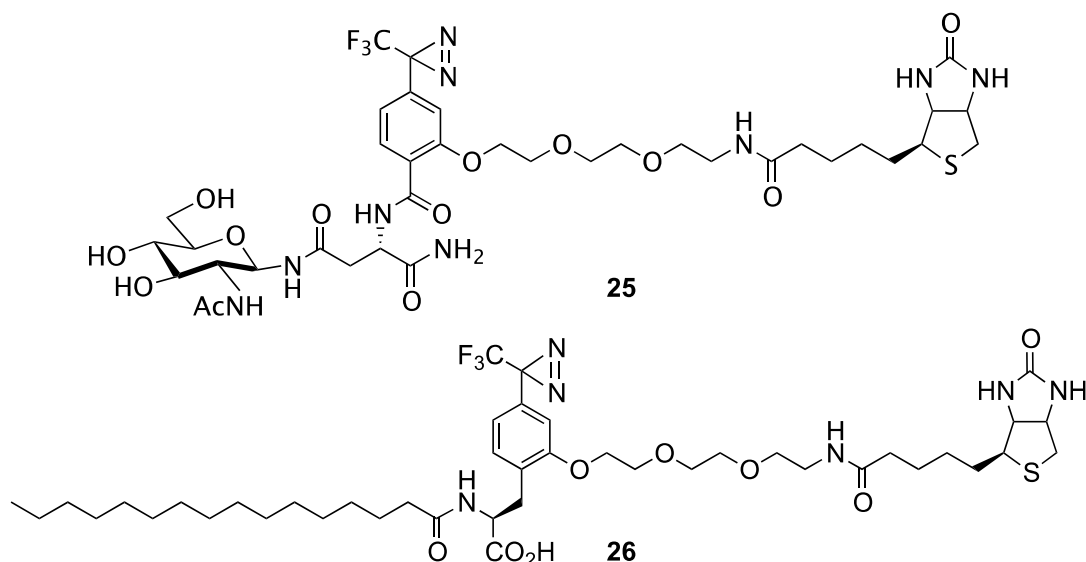


Figure 11. Biotinylated photo-probes of *N*-acetylglucosamine and palmitic acid

4-3. Fluorescent Tag

Aside from photobiotinylation, fluorescent agents have been used to select and identify labeled proteins. Fluorescent agents possess sufficient spatial and temporal resolution and are available in a variety of emission wavelengths that are suitable for molecular imaging. However, the low labeling yields in PAL approaches make in situ imaging of low abundant proteins difficult. Some examples of target detection by combining photocross-linkers and fluorophores follow. A probe using benzophenone coupled with biarsenical fluorescent dyes (**27**; Figure 12)⁵⁵ was developed to label the tetracysteine motif CCXXCC with high specificity and was used to capture and identify protein-protein interactions in living cells.⁵⁶ A phenyldiazirine-based photo-probe for aplyronine A (**28**),⁵⁷ an actin-depolymerizing substance of marine origin, possessing fluorescein incorporated into the side-chain portion clearly visualized actin to reveal its interaction, and a photo-probe of bisphenol A (**29**),⁵⁸ a common environmental endocrine disruptor, was synthesized, and the inhibitory activity of the derivatives was evaluated against the hypoxic response. Different peptidyl hydroxamates tethering diazirine and the Cy3 dye (**30**)⁵⁹ were designed to target metalloproteases and revealed unique labeling profiles correlating with various substrate peptides, indicating that this method could be extended to profile the substrate specificities of different enzymes. Alternatively, dual fluorescence-based target screening has been developed for selective detection of

small ligand-binding proteins by dual PAL (using benzophenone derivatives) in the presence of both active and inactive probes with different fluorophores.⁶⁰

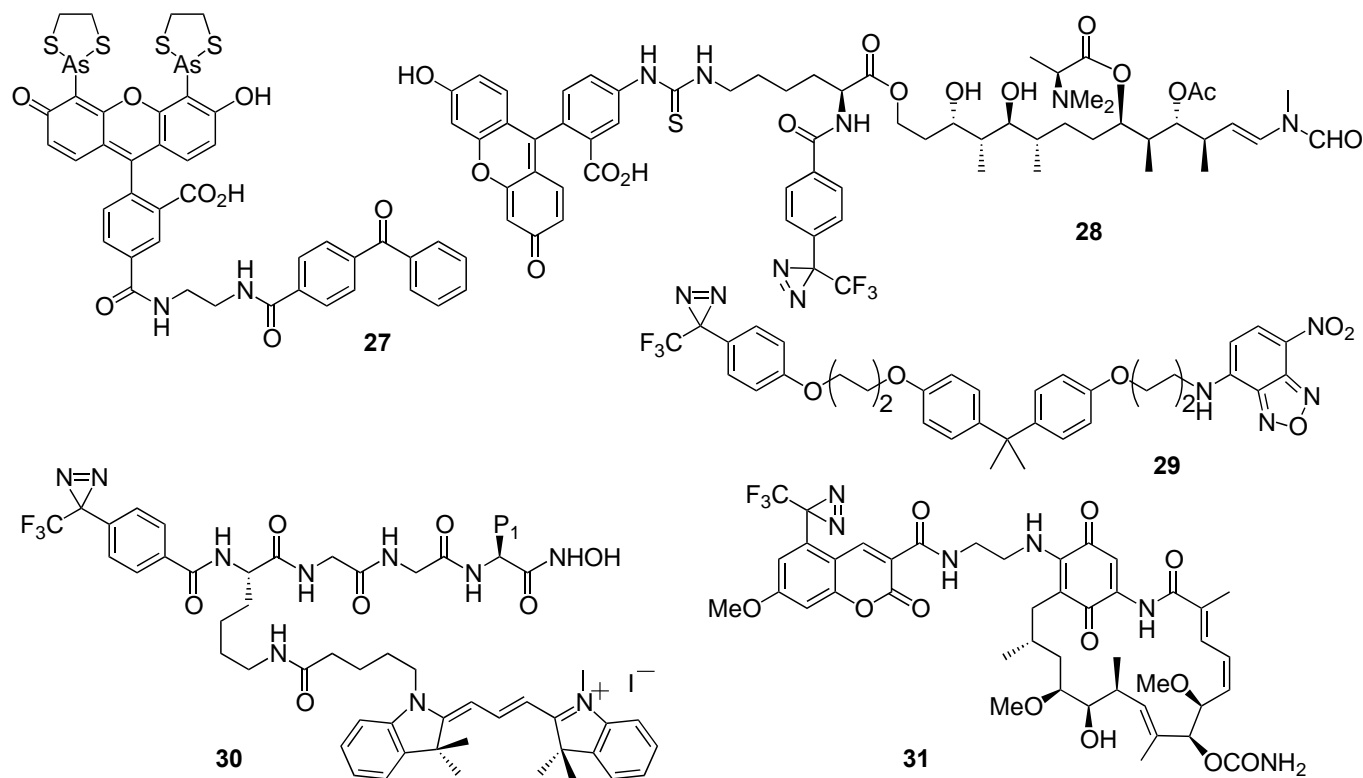


Figure 12. Photo-probes bearing a fluorophore

In general, however, due to the lower stability of fluorophores under irradiation or synthetic and/or physiological condition, their relatively bulky size, and their lower sensitivity compared to radioisotope and chemiluminescence detection, fluorescent methods have seen limited use in PAL studies. To overcome these significant disadvantages, a strategy for post-labeling with fluorophores has been developed wherein the target protein is captured with a photo-probe bearing a cleavable linkage or a clickable function (terminal alkyne or aliphatic azide)⁶¹ (Section 4-6). Alternatively, some fluorogenic dyes undergo dramatic fluorescence enhancement upon binding to proteins and are often applied for various in situ molecular/cellular imaging purposes with a high signal-to-noise ratio.⁶² These are mostly utilized in fluorescence-quenching systems. A similar fluorogenic property was recently reported for a diazirine-fused coumarin derivative (**31**; Figure 12).¹¹ Coumarin fluorescence was efficiently quenched by the diazirine moiety and was intensively recovered upon photolysis. Specifically, the cross-link-dependent fluorogenic labeling system was employed for the detection of heat shock protein 90 using a photoactivatable geldanamycin.

4-4. Fluorous Tag

Since perfluoroalkanes (R_F) have different hydrophobic properties than general alkyl groups, photochemical-based R_F tagging has been developed for highly selective and rapid purification of PAL-captured proteins by fluorous solid phase extraction (FSPE) and fluorous chromatography over fluorous silica. Zhang et al.⁶³ ($R_F = C_3F_7, C_6F_{13}$) and Grond et al.⁶⁴ ($R_F = C_4F_9, C_8F_{17}$) prepared perfluorinated phenyldiazirine substituted at the third carbon of the diazirine ring with an attached perfluoroalkyl chain (**32**; Figure 13) via different synthetic routes. A fluorous tag (C_6F_{13}) was incorporated into phosphatidylinositol 4,5-bisphosphate (**33**) to label ADP-ribosylation factor 1.⁶⁵ In addition to straightforward elution from the support, fluorous tags do not fragment during amino acid sequence analysis via MS/MS, which is greatly beneficial in proteomics. Fluorous tags avoid the problems in conventional biotin/avidin-based purification systems such as nonspecific adsorption to the avidin-immobilized support, incomplete elution, and complications from fragmentation during MS/MS analysis.

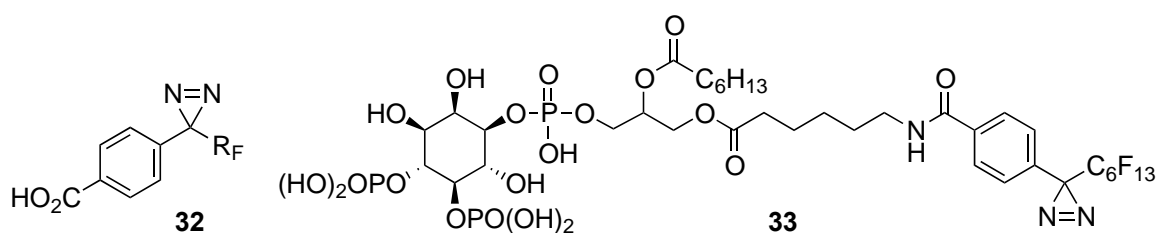


Figure 13. Perfluoroalkylated diazirines

4-5. Cleavable Tag

Biotin has been applied as a purification tag using an avidin-immobilized affinity support. The irreversibility of biotin-avidin binding under physiological condition permits enrichment of low quantities of labeled proteins as well as highly sensitive chemiluminescence detection using a streptavidin-HRP conjugate and has accelerated the profiling of a variety of labeled proteins. However, elution from the support requires harsh conditions, which often cause significant background noise due to nonspecific adsorption on the support and result in the identification of many candidates for the target protein. In addition, the presence of natural biotin and biotin-bound proteins in living cells often critically disrupts and complicates the target identification process. Toward addressing this crucial problem, immunobiotin, desthiobiotin, and monomeric avidin, among others, have been used to decrease the binding affinity. Furthermore, a cleavable function has been introduced between biotin and a diazirine photophore to allow mild elution of the labeled proteins and selective separation from intrinsically biotinylated proteins. The linkage is either enzymatically cleavable,⁶⁶ oxidation-sensitive (**34**),⁶⁷ reduction-sensitive (**35**),⁶⁸ nucleophile/base-sensitive, electrophile/acid-sensitive, or photoreactive (Figure 14).⁶⁹

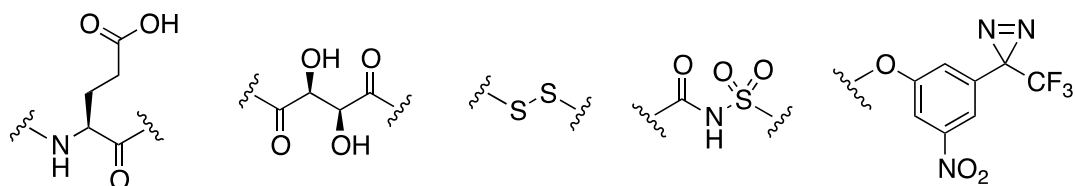


Figure 14. Cleavable groups used in photoaffinity labeling

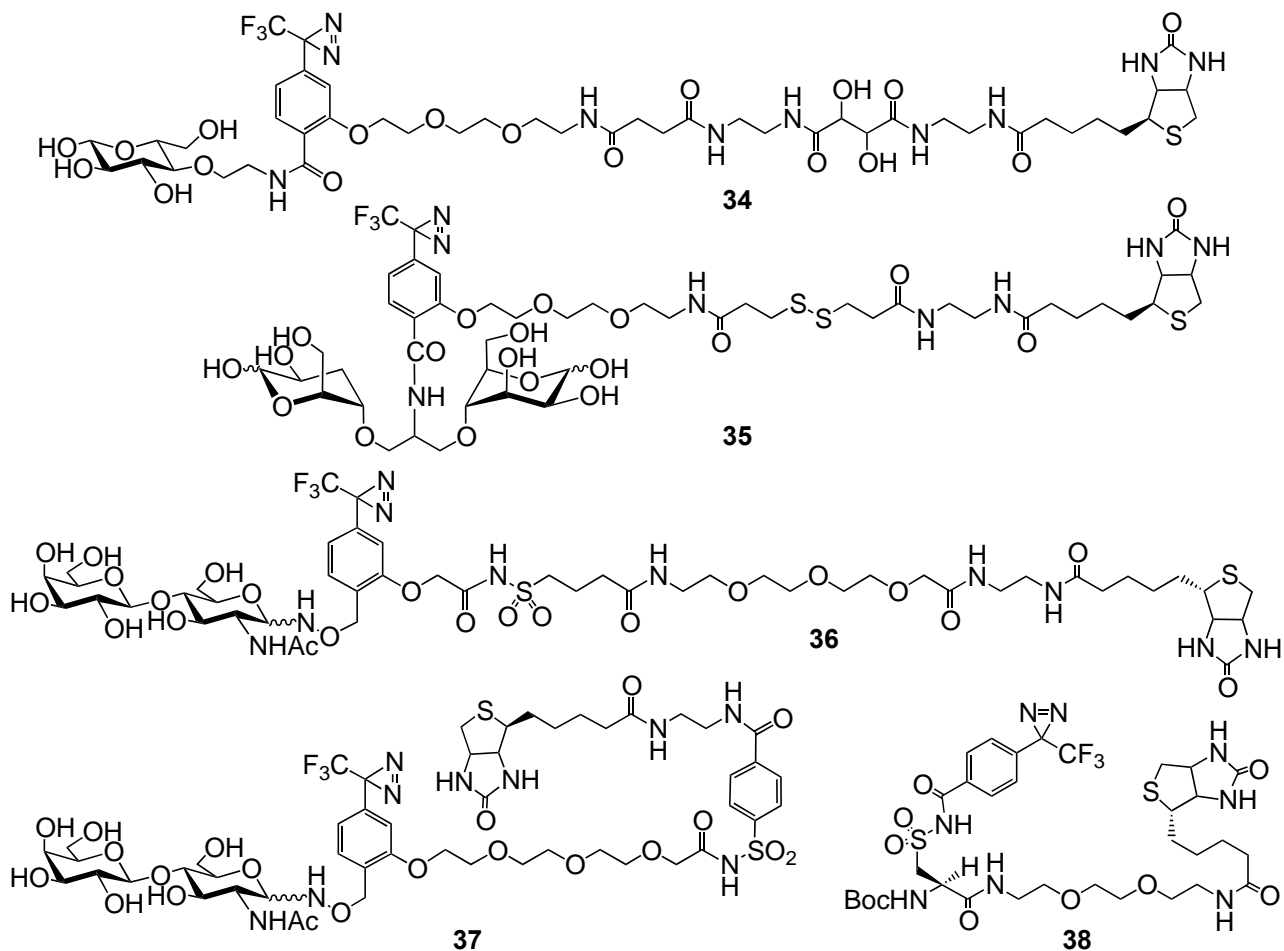


Figure 15. Examples of bio-probes bearing a cleavable unit

Acylsulfonamide, a nucleophile-sensitive linkage, was first applied to the biotin-avidin system for selective isolation of target proteins such as galactose-specific lectin and *Ricinus communis* agglutinin (RCA) using Gal β -1,4-GlcNAc (LacNAc) probes (**36**, **37**; Figure 15),⁷⁰ and glutamyl endopeptidase from *Staphylococcus aureus* V8 strain using a glutamate probe (**38**).³¹ Acylsulfonamide is very stable under general synthetic conditions and is applied for solid phase peptide synthesis as "safety-catch linker", but it is easily hydrolyzed after N-alkylation. For further MS analysis, the *N*-acylsulfonamide group was chemically stable when treated with 4-vinylpyridine, which is often used as an alkylating agent for the reductive alkylation of thiol groups. After S-alkylation with 4-vinylpyridine, the labeled protein was

trapped on an avidin-immobilized support and selectively and mildly eluted by N-alkylation with iodoacetic acid.⁷¹

4-6. Post-Tagging

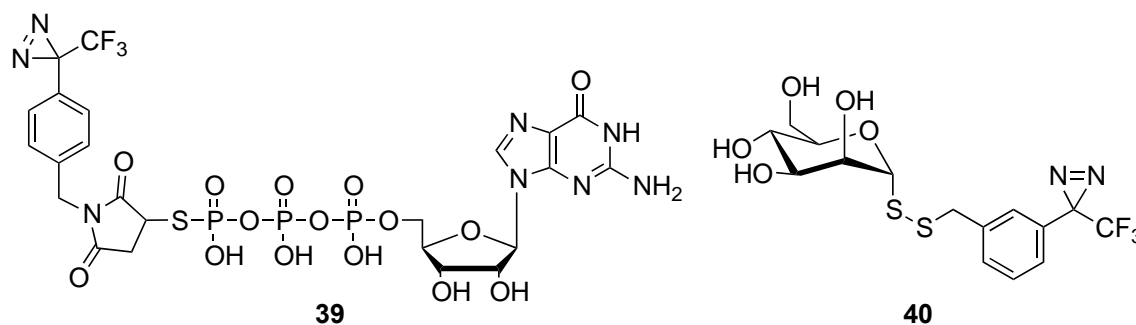


Figure 16. Bio-probes for post-labeling

A GTP probe modified with a phenyldiazirine at the terminal phosphate via a phosphorothioate linkage (**39**; Figure 16)²⁵ was synthesized and used to label H-Ras, a family of small GTPases. In this case, the P-S bond was easily hydrolyzed in an alkaline solution to produce a thiol group that was utilized for post-labeling of a biotin for high-sensitive detection of labeled H-Ras. On the other hand, Hamachi et al. reported a fluorescence-based saccharide sensing system.⁷² After cross-linking a photoactivatable saccharide to concanavalin A (Con A), a fluorophore was subsequently attached to a thiol group produced by the reductive cleavage of a disulfide bond between a phenyldiazirine and a saccharide (**40**). This strategy can introduce a photolabile fluorophore into the interacting region by post-labeling and reduce steric effects on specific ligand interactions. However, it requires protection of other thiol groups on the protein surface before post-labeling. In recent years, small ethynyl and azide clickable tags have been installed in the probe structure and have proven useful for the introduction of a variety of tags by post-labeling using bioorthogonal chemistry, which does not require the protection of functional groups (Figure 17).⁶¹ For example, a bis-azido cross-linker bearing both aliphatic and aromatic azides was developed and utilized for selective detection of aryl azide-based PAL-captured target proteins by attaching a fluorescent tag with an alkyl azide group through the Staudinger-Bertozzi ligation (**44**; Figure 17).⁷³ A combination of diazirine and clickable tag was also examined for detecting the binding target of fungal cyclodepsipeptide (**41**)⁷⁴ and β -D-glucopyranosyl 12-hydroxyjasmonic acid (**42**)⁷⁵ by post-attachment of rhodamine and biotin, respectively. Park et al. successfully identified two G-protein-coupled receptors, which cooperatively mediate ascaroside perception using a photobleaching fluorescent energy transfer assay system constructed with a clickable tag (**43**).⁷⁶ Recently, new types of bifunctional diazirine photophores were prepared by introducing these reporter tags onto the benzene ring (**45**)⁷⁷ or trifluoromethyl group (**46**,⁷⁸ **47**⁷⁹) of aromatic or aliphatic diazirines (**48**).⁸⁰

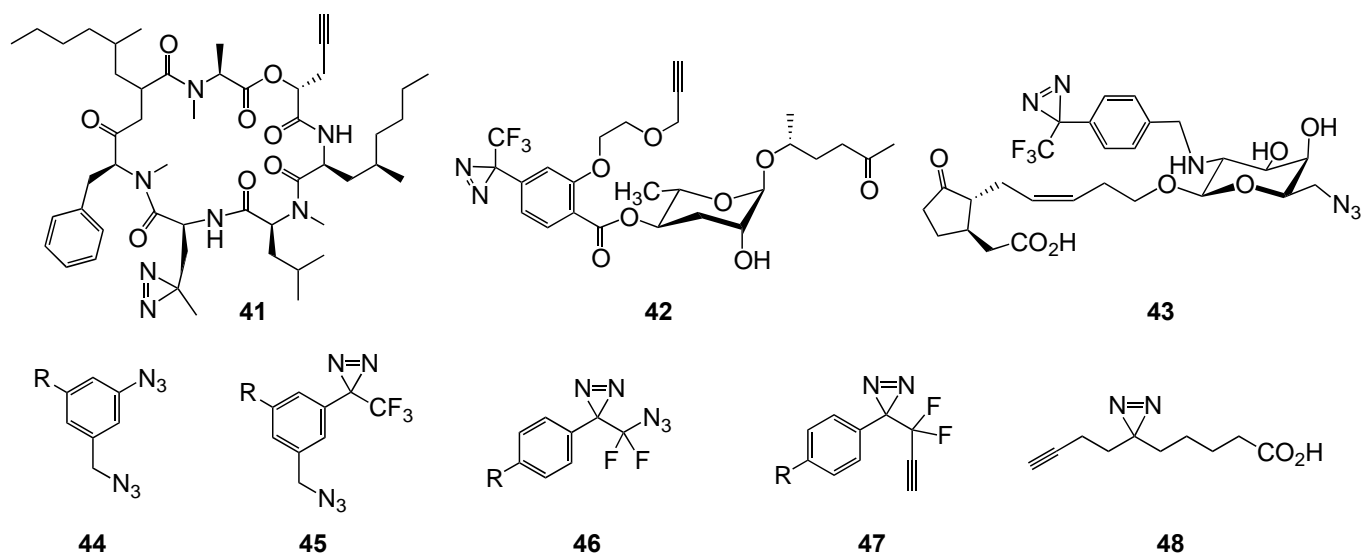


Figure 17. Multifunctional clickable photocross-linkers (44-48) and bio-probes (41-43)

5. OTHER TARGET DETECTION METHODS COMBINED WITH HIGH-THROUGHPUT TECHNOLOGY

5-1. Photoscreening of Potent Inhibitors

PAL-based target screening combined with a solid support modified with protein or ligand molecules has some advantages over conventional affinity methods. Stable covalent capture of noncovalently interacting molecules on the support is a major advantage that allows thorough washing to remove nonspecifically adsorbed materials from the surface. It is especially beneficial for detection of trace amounts of materials and weak-binding target molecules. Additional advantages of this method include facile handling of chemical treatments, parallel detection, and enrichment of infinitesimal amounts of labeled target.

PAL-based inhibitor screening has been demonstrated for interactions between D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Cibacron blue 3GA, a model ligand that binds the dinucleotide fold of proteins.²³ Cibacron blue 3GA was immobilized on the solid surface through a PEG spacer, and photoactivatable GAPDH was prepared by coupling thiosulfonate (**8**; Figure 3) with a cysteine residue located at the binding site. Upon photolysis, GAPDH was covalently captured on the surface by cross-linking to Cibacron blue 3GA. This method minimized nonspecific cross-linking compared to conventional methods based on the interaction between a protein and a small photoactivatable ligand, because the cross-linking should occur only in the binding site instead of nonspecifically on the protein surface. Addition of competitive inhibitors proportionally diminished the extent of GAPDH captured on the surface, making the approach useful for the screening of potential inhibitors. Protein fabrication has been directly performed on an inert polypropylene surface using

photoactivatable hydrophilic polymers by photoirradiation. These polymers were prepared by coupling reactions of polyamines such as polyethyleneimine, poly(L-lysine hydrobromide), or polyacrylic acid with epoxide (**49**; Figure 18) or sulfonamide (**50**) diazirine derivatives.⁸¹ Enzymes such as peroxidase, lysozyme, or pepsin were functional when trapped in the soft polymer cushion. Specifically, the use of a polymer bearing a hydrophilic spacer between the diazirine and polymer main chain (**51**)⁸¹ significantly increased the activity.

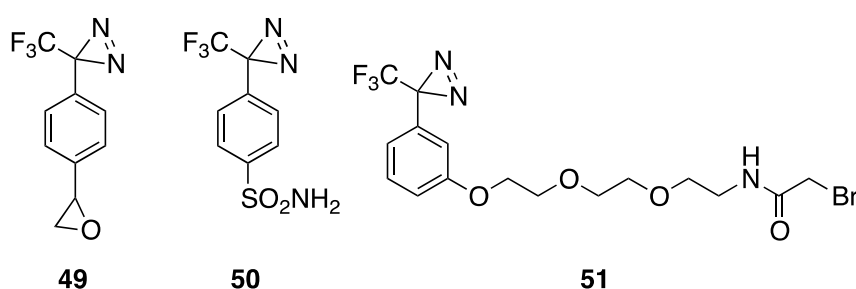


Figure 18. Diazirine derivatives for photoactivatable polymers

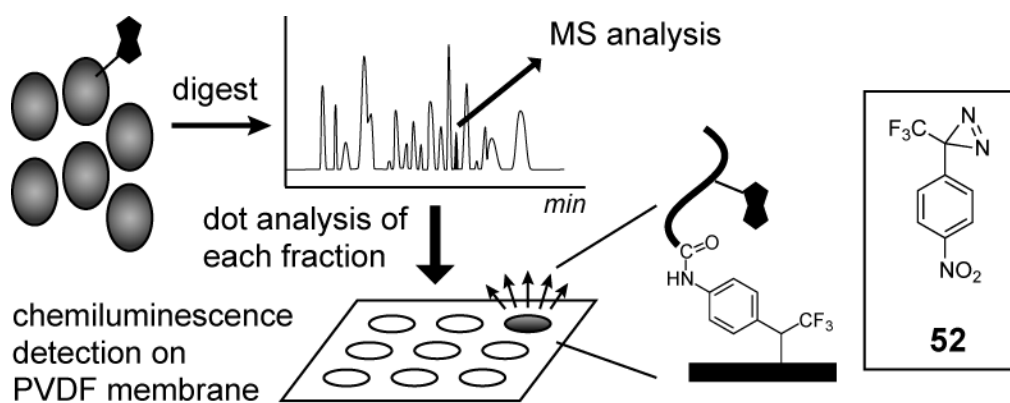


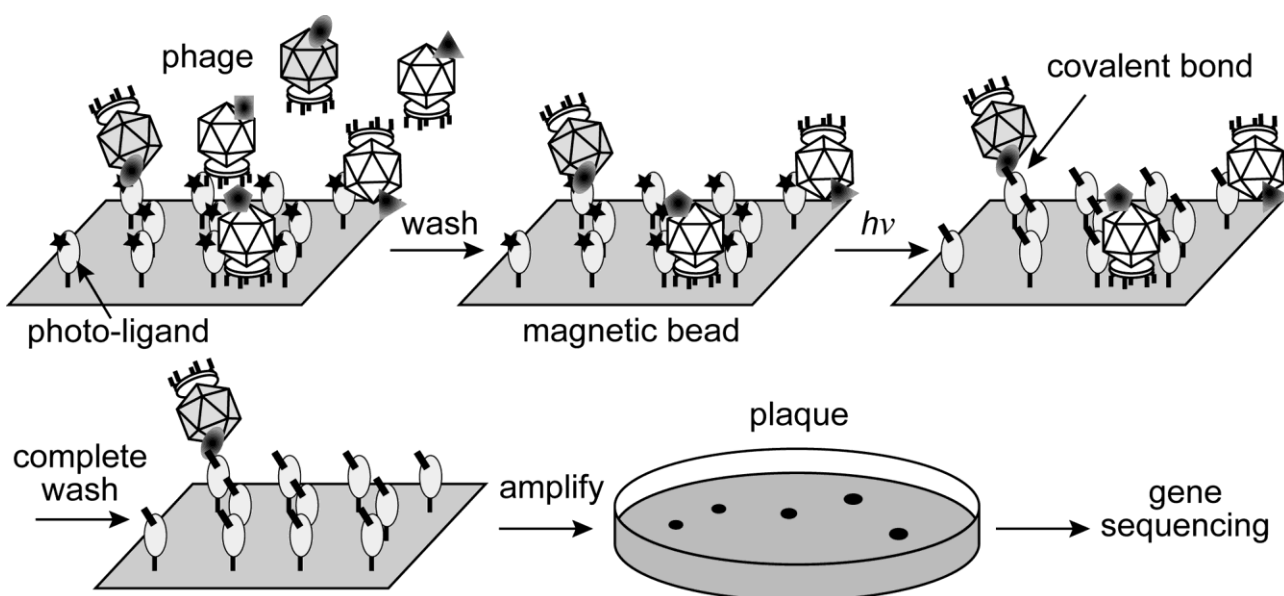
Figure 19. Dot blot identification of a biotinylated peptide captured on a photo-fabricated polyvinylidenedifluoride membrane for discriminating the corresponding HPLC fraction

A nitrophenyldiazirine derivative (**52**; Figure 19) was used for direct photochemical surface fabrication of a polyvinylidenedifluoride (PVDF) membrane.⁴⁹ Upon UV irradiation, nitro groups were covalently attached on the surface, and their subsequent reduction produced the aminophenyl membrane, which could be used to immobilize the peptide (Figure 19). Separation of the labeled and unlabeled fragments presents a serious problem for identification of the labeled site, as the latter are far more abundant. Therefore, it is very difficult to identify the target peptide via HPLC. In this system, after HPLC of the digestion product, each fraction was spotted on the aminophenyl PVDF membrane to immobilize peptides. The fraction containing the biotin-tagged peptide can be distinguished using highly sensitive

chemiluminescence detection on the membrane. The simple dot blot assay was applied to rapid identification of the GalT acceptor site.

5-2. Photo-Panning

Despite the ease of DNA amplification, it is impossible to copy proteins of interest directly for analysis. Therefore, identification of trace amounts of labeled proteins in low-abundance or low-affinity often requires cumbersome analytical processes. In addition to the highly selective purification techniques described above, display methods have been developed as another promising way to identify target proteins. During the last decade, phage display⁸² and some in vitro display techniques such as RNA displays,⁸³ in which the phenotype (the individual peptide/protein) is linked to its genotype (the coding sequence), are powerful tools for selecting peptides and proteins that bind with specificity to target molecules from random libraries. A trace target protein can be identified by sequencing its amplified genome; however, identification of low-affinity proteins was still difficult because these were easily lost during washing to remove nonspecifically adsorbed materials. Photoactivatable calmodulin binding peptide (CaM-BP) incorporating a diazirine photophore was synthesized and then immobilized on magnetic beads. After incubation with a phage library, photoirradiation established a covalent bond to phages displaying binding proteins/peptides, which allowed conduction of stringent washing steps. This photochemical bio-panning greatly enhanced the efficiency of target selection (Scheme 5).⁸⁴



Scheme 5. Strategy for biopanning of phage-displayed target-peptide by photocross-linking

6. COMBINATION OF PAL AND LC-MS/MS FOR LABEL-DEPENDENT PROFILING OF PROTEIN FRAGMENTS

To identify the labeled proteins, isolated proteins are digested with proteases such as trypsin and lysyl endopeptidase, and PMF analysis identifies candidates of target proteins. Because of the likelihood of contamination, amino acid sequencing of the labeled peptide must be determined by MS/MS analysis or Edman degradation along with the following immunological assay to verify the result. Unfortunately, finding the few labeled peptides in a complex digestion mixture is extremely difficult.

Affinity-based cross-linking with MS analysis is a powerful approach to investigate target identification and the interacting structure of proteins and their complexes.⁸⁵ Cross-linking experiments of proteins are evaluated to map the interaction surface by performing MS identification of the labeled peptide after enzymatic digestion based on the resulting proteolytic peptides. However, MS identification of labeled products can be hampered by the inherent complexity of the cross-linking reaction mixtures. Furthermore, the individual physical properties of each peptide fragment cause different isolation efficiencies due to loss by nonspecific adsorption onto the apparatus or contamination with nonspecifically adsorbed materials from the apparatus, which can be critical when dealing with peptides at the pico/femtomole scale. To find a labeled peptide in a digestion mixture, a number of strategies have been developed to facilitate the identification of cross-linked products by introducing discriminating properties or by enriching the peptide of interest using specific tags as described before. The strategies mainly utilize PAL probes bearing isotopic or fluorescent tags, which render the labeled target distinguishable from a large excess of unlabeled proteins upon separation on a SDS-PAGE gel, or bearing an MS tag to discriminate the target signal in MS analysis.

In particular in MS analysis, isotopic labeling of the proteome has gained popular acceptance for protein profiling because of its potential in the discrimination of target products from the enormous amount of unlabeled proteolytic digest. In chemical methods, isotope tags can be introduced into special functional groups within a protein such as primary amines, sulfhydryls, and carboxyl groups. Commonly, mixed isotope tags such as isotope-coded affinity tag (ICAT),⁸⁶ isotope-coded protein label (ICPL),⁸⁷ and isobaric tags for tandem MS such as relative and absolute quantitation (iTRAQ)⁸⁸ and tandem mass tag (TMT)⁸⁹ have emerged as potent tools for quantitative proteomics analysis. For PAL, some phenyldiazirine photophores incorporate stable isotopes (**53**; Figure 20) such as deuterium or carbon-13.⁹⁰ Recently, another deuterated phenyldiazirine bearing a perfluoroalkyl chain instead of common trifluoromethyl group (**54**)⁹¹ was synthesized for efficient profiling of the labeled products. The photophore includes three functional groups in the rather small structure: diazirine for capturing, R_F for purification, and an isotope for MS detection.

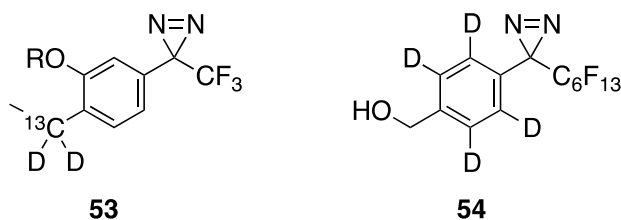
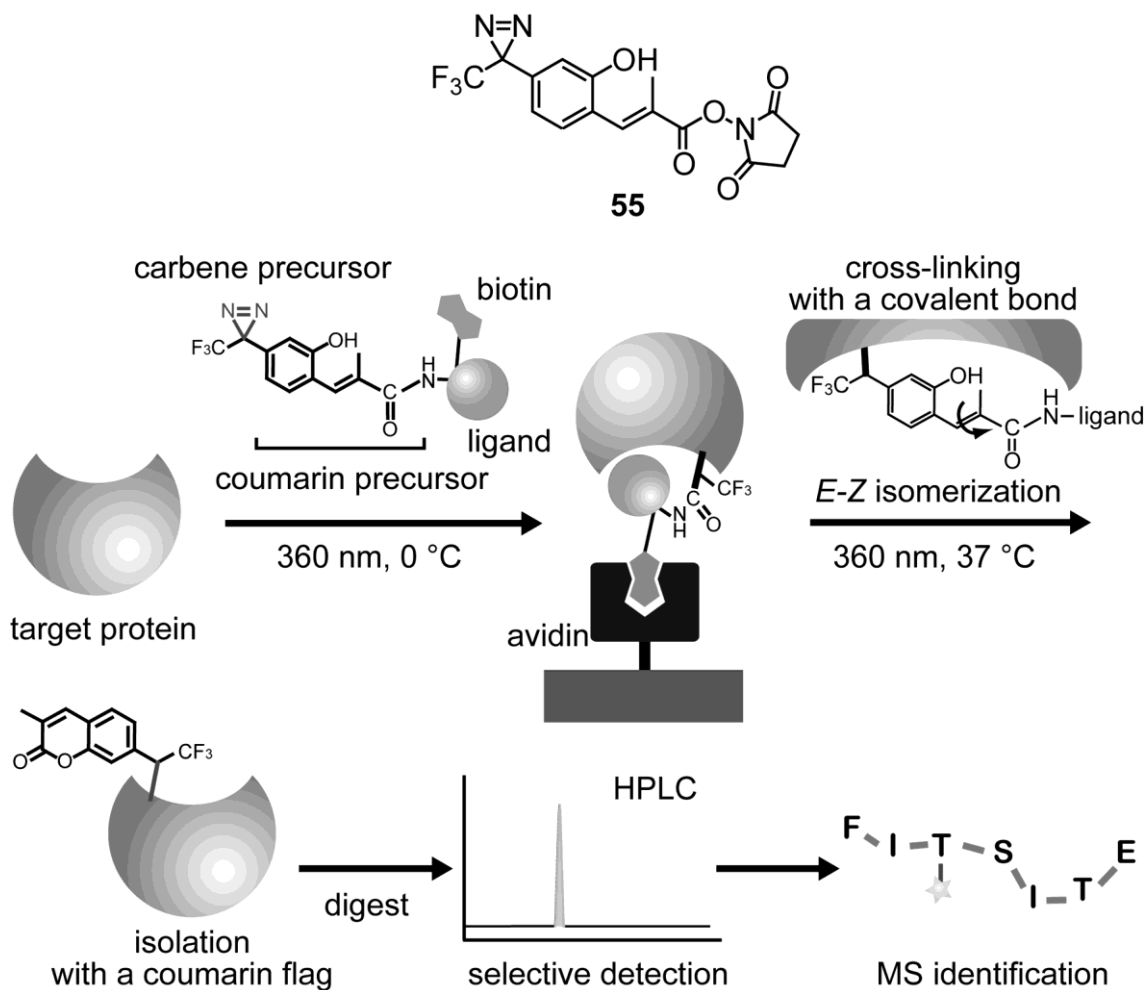


Figure 20. Deuterated phenyldiazirine derivatives

Scheme 6. Structure of fluorogenic diazirine bearing an *o*-hydroxycinnamate moiety and the outline of target peptide identification by a photochemical tag switching system

Recently, a novel affinity-based photochemical fluorogenic labeling method was developed utilizing diazirine bearing an *o*-hydroxycinnamate moiety (55; Scheme 6), which has been applied to image B-cells expressing surface anti-lysozyme using photoactivatable lysozyme (Photo-Lys).⁹² Upon brief photolysis after incubation with Photo-Lys, B-cells were visualized by coumarin emission. The photoreactive unit exhibits two photoreactions, photolysis of diazirine and *E-Z* photoisomerization of cinnamate, and the latter reaction induces the intramolecular substitution reaction to produce a coumarin ring. Consequently, this sequential reaction creates a coumarin fluorophore on the interacting protein while leaving the bait

lysozyme. Fluorescence of the coumarin tag incorporated at the interacting site allows the labeled peptide to be distinguished from the large number of unlabeled peptides in the HPLC analysis.⁹³ This peak can be directly identified by liquid chromatography (LC)-MS/MS analysis without purification. In addition, the coumarin tag is small, which is beneficial for maintaining high resolution during MS analysis of the labeled peptide. Furthermore, this tag is stable under denaturing conditions, digestion procedures, and collision-induced dissociation (CID) at typical energy levels; therefore, it could succeed in avoiding complex fragmentation by CID in MS/MS analysis.

The cross-linker unit (**55**) is multifunctional in a small structure exhibiting two kinds of photoreactions as described above, fluorogenicity, a capability for ligand introduction, and cleavability. Since the second photoreaction is controllable separately from the first photoreaction, the scissile function can be utilized for the selective isolation (enrichment) of the protein captured by the first photoreaction, which is much easier to handle than a peptide. In fact, the photochemical biotin-labeled vacuolar sorting receptor of soybean with a photo-probe of signal peptide (RAFY, the sorting determinant peptide) was selectively captured on the avidin-support,⁹³ gently eluted via the second photoreaction using <360-nm irradiation at 37 °C, and successfully separated from intrinsic biotinylated proteins. Thus, the scissile function of biotinylated bioprobes should be greatly advantageous for mild elution of labeled products from the robust biotin-avidin support, but the detection tag was also forfeited in the process. In the new PAL system, however, a coumarin fluorophore is concurrently formed on the labeled protein when cleaved from the biotin, and the coumarin tag differentiates the labeled peptide from numerous nonlabeled peptides with high clarity and sensitivity. This photochemical tag switching process dramatically enhanced efficiency of labeled protein/peptide identification and revealed the binding domain for the sorting determinant peptide in its membrane receptor within a short period.

7. CONCLUSION AND FUTURE DIRECTIONS

Many cellular proteins do not act in isolation but are embedded into cellular networks and are securely connected physically and functionally with other cellular components. In addition, the several hundred types of cells that comprise different organs constitute different physical and functional environments. Although crystallography and NMR spectroscopy are useful in solving 3D protein structures with high resolution in the stable interacting state, these structural analyses of such complex biomolecular interactions such as integral signaling complexes are extremely difficult, and the throughput is rather small since the coverage suitable for crystallization is limited. Alternatively, specific photocross-linking methods using a photochemical probe can be applied to various types of interactions and they provide structural information about the binding site. Establishment of the fundamental science and technologies

of useful diazirine photophores, such as derivatization and molecular probing, has contributed to the recognition of PAL as a useful and versatile method for target identification. Recent progress in PAL with respect to the aspects of its multifunctionality and sophisticated strategies have significantly reduced the period for target identification and resulted in a high success rate of target identification. PAL-based binding analysis with structural information will offer a more precise understanding of the complex recognition mechanisms among biological molecules. Although additional improvements may be required for these methods to become a generally applicable technique in proteomics, development of innovative strategies for the discrimination of cross-linked products and facilitation of MS detection with advances in the available computer software for automated MS data analysis will establish PAL as a ready-to-use technique in the field of life sciences.

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