DOI: 10.1002/ejoc.200701069

Recent Progress in Diazirine-Based Photoaffinity Labeling

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Keywords: Photoaffinity labeling / Chemical biology / Bioorganic chemistry / Structure–activity relationships / Diazirine

As a result of recent developments in molecular biology, the investigation of biofunctional machinery at ligand-accepting interfaces has become one of the challenging and important subjects in the post-genome field. The technique of photoaffinity labeling has become increasingly appreciated as a powerful methodology for this purpose. This microreview focuses on the synthesis of 3-phenyl-3-(trifluoromethyl)diazirine, one of the most promising photophores, and its application to biomolecules.

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Introduction

Elucidation of protein functions on the basis of structure–activity relationships in order to reveal the mechanisms of homeostasis functions in life is one of the greatest interests of scientists. In the human body, many proteins are activated and/or inactivated by various ligands to maintain homeostasis. Understanding the mechanism of molecular interactions between small bioactive ligands and proteins is an important step in rational drug design and discovery. For these purposes, a genetic approach provides an efficient and indirect route for pinpointing functional amino acids within proteins through deletion or mutation of the native amino acid alignment in a proteins. When the target protein can be expressed in large quantities, 3D structural determinations, involving NMR spectroscopy and homology modeling, are a powerful and direct approach for analyzing the three-dimensional structures of proteins at the atomic level. Chemical methods, which are fundamental in chemical biology, provide an alternative route for the direct identification of target proteins in biomolecule mixtures as well as their ligand binding site structures, because these analyses are based on the affinity between the ligand and the target protein (Figure 1). Affinity-based chemical modification introduces a useful tag for analyzing the target protein. For example, single-molecular imaging with a fluorophore[1] visualizes target biomolecules in complex systems for imaging of the localization of biomolecules and processing the flow of bioactive compounds in cell compartments. Affinity labeling[2] has a limited role for specifically attaching the desired tag on the target protein, because the method requires

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the presence of nucleophilic residues near to a ligand binding site in order to prevent nonspecific introduction of the tag at a site different from the binding site or onto other coexisting biomolecules. Recent progress in molecular biology, though, has encouraged site-directed labeling using cysteine mutants to overcome this problem.[3]

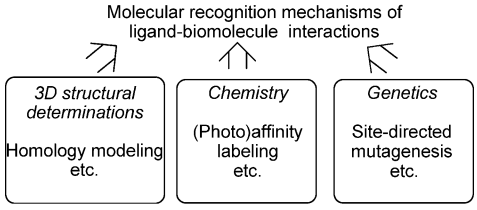

Figure 1. Approaches to the structural analysis of ligand–receptor interactions.

Photoaffinity labeling[4] has greatly increased the capability of specific tagging. Photochemically generated highly reactive species introduce covalent bonds between ligand and protein in a nonselective manner, and any amino acid in the binding site can be tagged (Figure 2).

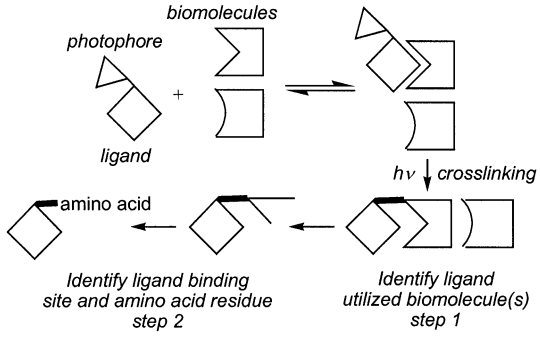

Figure 2. Schematic representation of photoaffinity labeling.

Successful applications of photoaffinity labeling include the identification of target biomolecules in crude extracts with the aid of radioisotope-labeled probes as highly sensitive detection tags (step 1 in Figure 2). The covalent bond fixes the tag to the contact point even though affinity has been destroyed by the denaturation conditions, which allows further sophistication in detailed structural analysis, and the detection of tagged components may reflect the status of conformational changes in target molecules. Our previous study on β-1,4-galactosyltransferase demonstrated that photoaffinity labeling can reveal conformational change during the enzymatic reaction. The enzyme uses two substrates – UDP-galactose (**1**; Figure 3) as a donor and *N*-acetyl-D-glucosamine (GlcNAc, **2**) as an acceptor – to generate *N*-acetyllactosamine (**3**). Specific photoincorporation of photoreactive GlcNAc derivatives was achieved only at 37 °C in the presence of UMP, which is a UDP-Gal analogue. The results indicated that the two substrates should be orientated close together in the active site to promote a conformational change in the enzyme structure.[5] Recently it was reported that dynamic structural investigations on the torpedo nicotinic acetylcholine receptor could be performed by time-resolved photoaffinity labeling.[6]

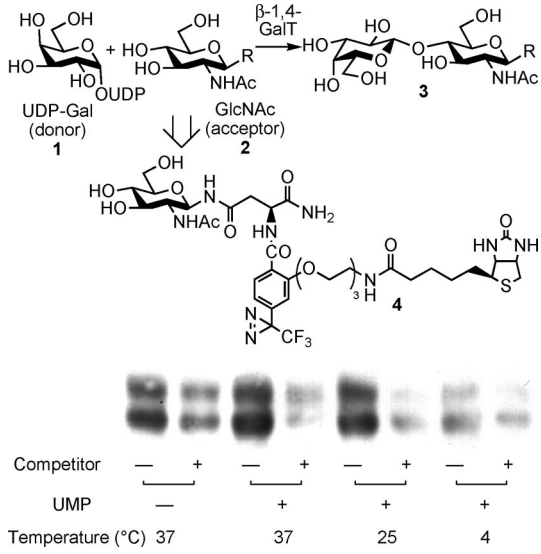

Figure 3. Molecular dynamics of β-1,4-galactosyl transferase (β-1,4-GaltT) with photoaffinity labeling with compound **4**.

On the other hand, there are few examples of photoaffinity labeling to identify the ligand binding site and labeled amino acid (step 2 in Figure 2), because the identification of photolabeled components has been hampered by low photoincorporation yields.[4,7] There are many options in photoaffinity labeling experiments: choice and synthesis of the photophore, photolysis conditions, and choice of tags for the identification of biocomponents.

As mentioned above, both organic chemistry (the preparation of the photophore and ligand modification) and biochemistry (handling of labeled components), are needed in order to perform photoaffinity labeling experiments.

This microreview focuses on the fundamentals of photoaffinity labeling experiments and on the recent developments in photoaffinity labeling with 3-phenyl-3-(trifluoromethyl)diazirine, one of the most promising photophores, and its applications for the effective analysis of ligand–biomolecule interactions.

a) Photophore Synthesis

i) Advantages of the Diazirine Photophore

It is important which photophores are used for effective photoaffinity labeling (Figure 4). Typically, an aryl azide, a benzophenone, or an 3-aryldiazirine is used. Aryl azides are photoactivated below a wavelength of 300 nm, which sometimes causes damage to biomolecules, and generate nitrenes[8] as active species, which sometimes rearrange to keteimines as undesired side products.[9]

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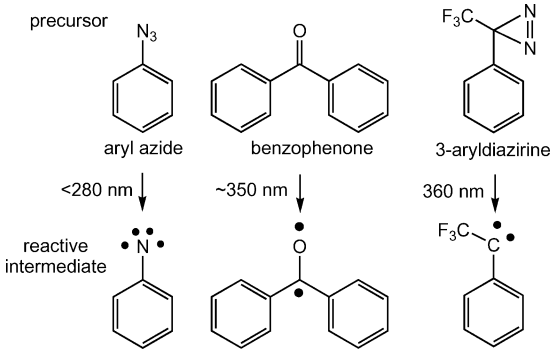

Figure 4. Photochemical reactions of major photophores for photoaffinity labeling.

Benzophenones[10] are photoreactivated with light over λ = 350 nm and generate reactive triplet carbonyl states. These regenerate ground-state carbonyl compounds and so benzophenones are reusable for photolabeling, although the photophores sometimes need long photoirradiation times for labeling.

3-Aryldiazirines[11] are also photoreactivated with light over 350 nm and generate carbenes, which are more highly reactive species than other photophores, rapidly forming cross-links to biomolecules with short photoirradiation times. It has been reported that the photolysis of diazirines can cause diazo isomerization, giving undesired intermediates in photoaffinity labeling. Diazo isomerization can be suppressed by introduction of a trifluoromethyl group into a diazirinyl three-membered ring.[12]

Comparative irradiation studies of these three photophore types in living cells suggested that the irradiation needed for the generation of active species from azide and benzophenone caused cell death because long irradiation times are needed to incorporate the photophores into cell membrane surface biomolecules. On the other hand, a carbene precursor – 3-phenyl-3-(trifluoromethyl)diazirine (TPD) – did not promote cell death in the generation of active species, but other photophores (i.e. γ-secretase) are sometimes utilized in photoaffinity experiments in vitro.[13] Although these results indicated that photoaffinity labeling with TPD has the most promising nature for investigation of labeled components in the cell, the applications are not as many as with other photophores, because synthetic construction of the TPD skeleton is not as easy as with other photophores.

ii) Efficient Synthesis of TPD

Synthesis of the TPD three-membered ring required at least five steps from the corresponding aryl halide derivatives (Figure 5, A). A tether should be introduced onto the benzene ring to connect to the ligand skeleton. Previous preparations of TPD derivatives had preinstalled suitable tethers before construction of the diazirine moiety, but it seems that the three-membered diazirinyl ring is not stable towards many organic reactions. The repeated construction of a diazirine moiety for each derivative is a drawback for application of the photophore for photoaffinity labeling. Our breakthrough work on "post-functional" adaptation of diazirinyl compounds[14] revealed that the trifluoromethyl-substituted three-membered ring was stable under many organic reaction conditions. Although the 3-(trifluoromethyl)diazirinyl moiety is categorized as an alkyl substituent, polarization means that the quaternary carbon atom is slightly positively charged, so the moiety is less activated towards electrophilic aromatic substitution than its unsubstituted counterpart.[15] We first selected the *m*-methoxy-substituted TPD **5** (Figure 5) as the mother skeleton, because: 1) the methoxy group strongly activates for electrophilic aromatic substitution, 2) the orientation of the substitution favors the *o*-position against the methoxy group, because the *p*-position is sterically hindered by the 3-(trifluoromethyl)diazirinyl moiety, and 3) demethylation of *m*-methoxy-TPD was easier than for *p*-methoxy-TPD, and realkylation of the demethoxyated group was utilized for introduction of the tag (Figure 5, B).

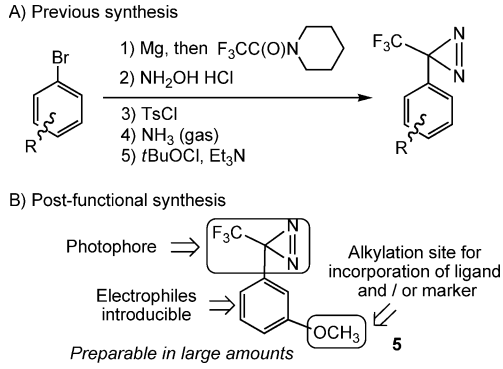

Figure 5. Prior synthetic routes (A) and post-functional synthesis routes (B) for TPD derivatives.

Compound **5** smoothly underwent the introduction of a nitro group as a chromogenic tag in the usual manner without decomposition of diazirinyl ring **6** (Figure 6).[14b] This result suggested many electrophilic reactions for compound **5**. The iodinated derivative is a useful compound for radioisotope introduction. Iodination of compound **5** was achieved 1) via thallic derivatives, 2) via alkyltin derivatives, or 3) with hypervalent iodine reagent.

Friedel–Crafts alkylation of **5** with dichloromethyl methyl ether, followed by hydrolysis, afforded aldehyde derivative **8**,[16] which had previously been prepared by preinstallation of an aldehyde group before diazirine construction.[17] Aldehyde **8** was easily converted into alcohol **9** by treatment with $NaBH_4$/methanol, into carboxylic acid **10** by treatment with manganese, and into cinnamic acid derivatives **11** through Wittig reactions with stable ylides in the usual manner.[16] (Wittig reactions with unstable ylides and **8** had very low yields because of the strongly basic conditions.[18])

Friedel–Crafts acylations of compound **5** are also widely utilized. These reductions were previously one of the most incompatible reactions for TPD derivatives, with the presence of the easily reducible nitrogen–nitrogen double bonds.

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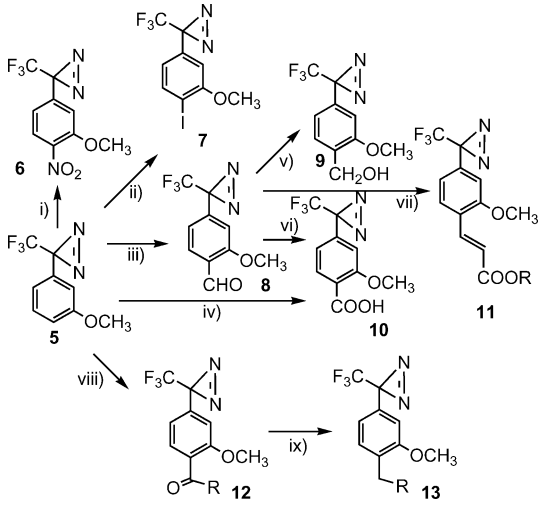

Figure 6. Chemical conversions for aromatic substitutions of compound **5** with post-functional synthesis. i) HNO_3, $(CH_3CO)_2O$, ii) I_2, bis(trifluoroacetyl)phenyl iodinate, iii) Cl_2CHOCH_3, $TiCl_4$, iv) $(CF_3CO)_2Tl$, then CO, $PdCl_2$, v) $NaBH_4$, vi) $(n\text{Bu})_4NMnO_4$, vii) $Ph_3CCHCOOR$, viii) RCOCl, $AlCl_3$, ix) Et_3SiH, TFA.

We tried to reduce the aryl carbonyl group to methylene components with various reactions – $LiAlH_4/AlCl_3$, tosylhydrazine/$NaBH_3CN$, and Wolff–Kishner reduction – but these conditions promoted decomposition of the diazirinyl ring. In the course of investigation, however, reduction with triethylsilane/trifluoroacetic acid was utilized for alkylphenone-type TPD derivatives **12** and the reaction was applied for the synthesis of the photoreactive fatty acid analogue **13**.[19]

The *m*-methoxy group was hydrolyzed with BBr_3 to provided phenolic derivative **14** (Figure 7) more easily than in the case of *p*-methoxy substitution.[14a] Compound **14** was used as starting material for the remethylation product **15** by treatment with radiolabeled methyl iodide, for enzymatic or chemical iodination to afford **16**, [14] and for *O*-alkylations to give **17** or **18**,[20] allowing for the biotinylation labeling described below.

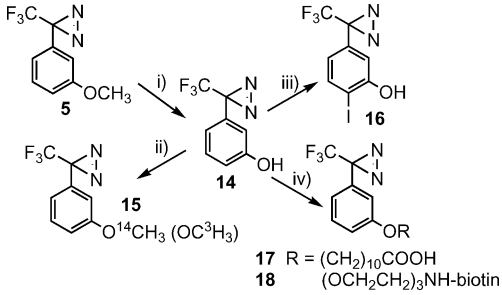

Figure 7. Chemical conversions for aromatic substitutions of phenoxy TPD derivatives **14** with post-functional synthesis. i) BBr_3, ii) $^{14}CH_3I$ or C^3H_3I, iii) NaI, Chloramine T, iv) $Br(CH_2)_{10}CO\text{-}OCH_3$, then aqueous NaOH.

Although the methoxy group was helpful in the aromatic substituent reactions, it sometimes caused reduced activity in the targeted biomolecules. We attempted post-functional derivatization for unsubstituted TPD **19** under various conditions (Figure 8). As mentioned previously, the diazirinyl moiety acts as a more electron-withdrawing group than its unsubstituted counterpart, but it was reported that the TPD ring was easily decomposed in the presence of strong acid over 25 °C.[21] $TiCl_4$ in CH_2Cl_2, which is a suitable promoter for the alkylation of compound **5**, does not promote the formylation of **19**.[22] These observations suggested the use of stronger acid for electrophilic aromatic substitution at low temperature. One such stronger acid, trifluoromethanesulfonic acid (TfOH) with SbF_5 or $TiCl_4$ and without solvent, was easily able to afford the desired product **20** without decomposition of the diazirinyl ring at 0 °C. It was also observed that the reactivity of the acyl/alkyl donor is also important for aromatic substitutions for unmodified TPD **19**. The reaction promoted the simple asymmetric preparation of photoreactive L-phenylalanine compound **21**.[23]

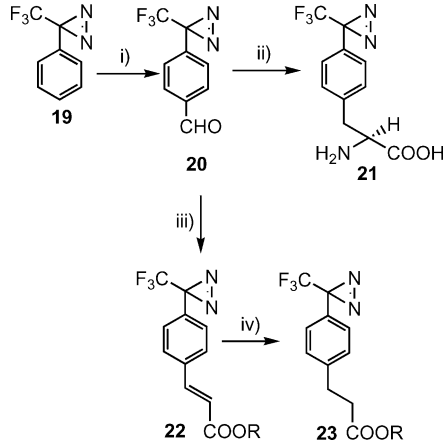

Figure 8. Post-functional synthesis of simple TPD **19**. i) Cl_2CHOCH_3, TfOH, ii) $Ph_2C{=}NCH_2COOtBu$ then TFA, iii) $Ph_3CCHCOOR$, iv) H_2, Wilkinson's catalyst.

Selective reduction of the carbon–carbon double bond in these TPD derivatives to give alkane systems is problematic. The TPD nitrogen–nitrogen double bond was destroyed in the presence of a heterogeneous catalyst (Pd/C under hydrogen for over an hour).[24] On the other hand, hydrogenation of **22** with Wilkinson's catalyst, a homogeneous catalyst, enabled selective hydrogenation of the carbon–carbon double bond over the TPD nitrogen–nitrogen double bond over 10 hours to afford **23**.[25]

These findings suggested building the diazirinyl skeleton first, and then introducing substituent groups to be used as tethers or tags. These "post-functional" derivatives from mother compounds have been produced not only by our research group[26] but also by other researchers.[27]

Many of these reactions are incompatible with the other azide and benzophenone photophores. These shorter methods for photophore preparation have prompted the routine use of the diazirinyl photophore for photoaffinity labeling experiments.

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b) Photoreactions

i) Ligand Concentration

One of the best sets of conditions for photoaffinity labeling experiments involves the addition of stoichiometric amounts of labeled reagents to a biomolecule, because the technique involves the binding site with labeled reagents, and excess reagents sometimes cause serious unspecific labeling at the expected position. Figure 9 shows photoaffinity labeling of erythrocytes with different amounts of biotinylated photoligand **24**. Excess photoactive ligand caused less inhibition of photolabeling with the natural ligand glucose. The results indicated that stoichiometry is important for photoaffinity labeling. It is preferable to set the concentration of the photoactive ligand, for which the affinity parameters for the target biomolecule (K_m, K_i, K_d, etc) are already known, 10 times higher against the affinity parameters to complete the ligand–biomolecule complex.[5a]

ii) Side Reaction

It was reported that the TPD was photolyzed with a black light lamp (≈ 350 nm) to generate the corresponding carbene.[12,28] Diazo isomerization was also reported as a side reaction for photoaffinity labeling (Figure 10). The diazo isomer easily generated a carbene on use of a shorter wavelength (305 nm)[29] or with longer irradiation with a black light.[12] These photolysis experiments were performed for ligand concentrations over 10 mM. On the other hand, actual photolabeling for biomolecules was performed at less than 1 mM. ^{19}F NMR studies of photodecomposition for compound **25** in 1 mM aqueous solution with a 15 W black-light lamp revealed that diazo isomerization is not a serious side reaction over 10 min (Figure 10, B). Serious diazo isomerization was observed at 10 mM, but smooth photolysis was observed after re-irradiation after the isomerized sample had been diluted to less than 1 mM. These results were consistent with actual photolabeling, of L-amino acid oxidase with 0.01 mM of **28** and chymotrypsin with 0.2 mM of **29**. There were no differences between irradiation with 350 nm light alone (Figure 11, B, lanes 1 and 3) and irradiation at 350 nm followed by 302 nm (Figure 11, B, lanes 2 and 4).[30]

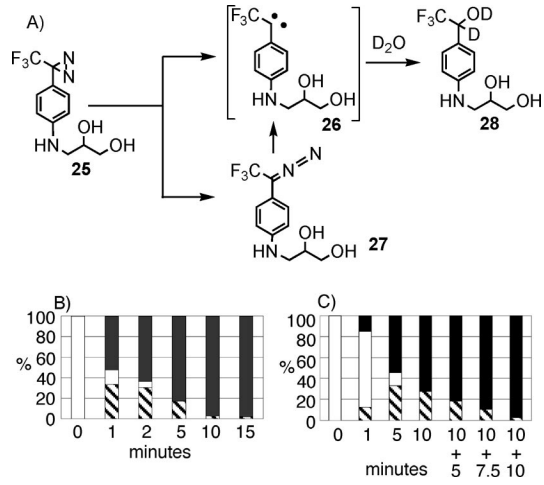

Figure 10. A) Photolysis of TPD derivative **25**, B) Contributions of chemical species with black light irradiation and TPD analogue **25** at 1 mM. Diazirine **25**, diazo **27** and adduct **28** are represented as an open column, shaded column and closed column, respectively. C) Contributions of chemical species with black light irradiation and TPD analogue **25** at 10 mM for 10 min, and subsequent dilution to 1 mM. The representations of each chemical species are the same as in B).

iii) Labeling Efficiency

Even though photolabeling conditions were set up as described above, photolabeling efficiency for the generation of covalent bonds between photophores and biomolecules was always less than 10%. It depends on many factors – orientation of photophores, transmittance of the photolabeling mixture, quenching of generated carbene by water and af-

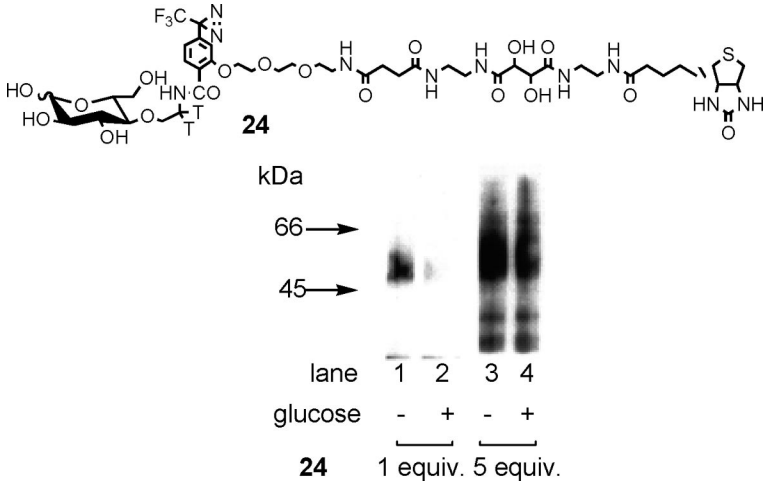

Figure 9. Photoaffinity labeling glucose transporter with different amounts of compound **24**.

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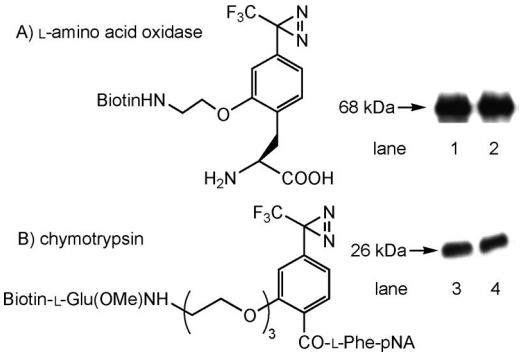

Figure 11.Photoaffinity labeling of L-amino acid oxidase (A) and chymotrypsin (B) with compound **28** (10 μM) and **29** (200 μM), respectively. Photolysis proceeded with black light (lanes 1 and 3) for 10 min, and also with black light for 10 min followed by light at 302 nm for 8 min (lanes 2 and 4). Photolabeled proteins were subjected to SDS-PAGE followed by chemiluminescence detection for quantitative densitometry analysis.

finity of the ligand etc. It was sometimes observed as near ~30% efficiency for high-affinity biomolecules (i.e., ion channels, receptors $K_d < 10^{-9}$ M).[31] This is no problem for the detection of labeled components, because highly sensitive methods (radioisotopes, etc) could be applied to the detection, but this low labeling efficiency hampered the identification of the labeled portion in biomolecules. Several approaches to solve these difficulties have been examined and are summarized in the next section.

c) Progress in Labeled Site Identification

To identify the labeled regions of target biomolecules, combinations with specific purification methods for labeled components are needed (Figure 2). Details are described in recent reviews.[4c,4e] Here we briefly describe the fundamentals of several concepts.

i) Immunological Methods

It is well known that antigen–antibody interactions show very specific recognition behavior, and generation of antibodies to biomolecules has been well studied. Recent developments suggest the preparation of an antibody against part of a target biomolecule (i.e., a selected peptide sequence). Specific antibodies that recognized particular parts of the target biomolecules were used for photolabeled mixtures in which the antibodies recognized the labeled region, which could be co-immunoprecipitated corresponding to the labeled and unlabeled components. When a radioisotope was introduced into the photophore, the labeled region was identified as an antigen peptide sequence. For example, Nakayama et al. clearly identified 1,4-dihydropyridine-binding regions for skeletal muscle Ca^{2+} channels.[31] It is very important that researchers be able to predict which part of the sequence should be used as the antigen.

Recently, antibodies for small molecules have been well developed. Similarly to antibodies to biomolecules, an antibody to the ligand skeleton was developed to reveal the labeled region directly. Photoreactive galactosylceramide **30** was recognized efficiently with anti-galactosylceramide antibody. These results indicated that the combination of photolabeling and immunological techniques was useful to identify the labeled region (Figure 12).[32]

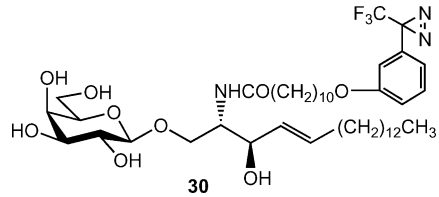

Figure 12. TPD derivatives of galactosylceramide.

ii) Chemical Tags in Mass Spectrometry

Radioisotopes have been utilized to detect labeled components for over three decades, because high radioactivity helps in the detection of photolabeled components, but the synthesis of radioactive TPD derivatives is not easy, because limited numbers of radiolabeled reagents are available for synthesis. One methodology was to introduce radiolabeled iodine into the benzene ring to afford TPD compound **31** (Figure 13), which was synthesized from the corresponding amino derivatives by diazotization followed by iodination. Iodine was also introduced with NaI/chloramine T or KI/hydrogen peroxide to afford phenolic TPD derivatives **32** and **33**. Less highly activated TPD derivatives for electrophilic substitutions were used to exchange between organic thallium[14a] or tin[27] and molecular iodine to afford **34**, as well as hypervalent iodine,[15] but labeled iodine-125 is one of the most difficult radioisotopes to handle because it has a short half-life. It is therefore useful to use radiolabeled tritium or ^{14}C for this purpose. Both radioisotopes have long half-lives and are more easily handled than iodine. Tritium is easily introduced into **35** by exchange of cold iodine and tritium gas in the presence of Pd/C over one hour. Phenoxy TPD **14** was easily converted into both tritium-labeled **36** and ^{14}C-labeled **37** by demethylation and remethylation with radiolabeled methyl iodide.[14a]

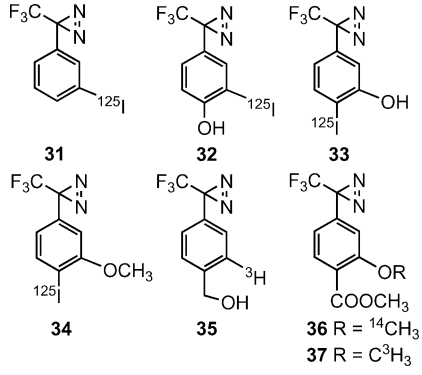

Figure 13. Radioactive TPD derivatives.

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The limitations of the specific (radio)activities of tritium and ^{14}C reagents are sometimes problematic. Furthermore, it is frequently observed that the yields of radioisotope synthesis are lower than those of the corresponding cold reagents, and the handling and storage of radiolabeled compounds are greatly hampered. Radiolabel-free synthesis of compounds that contain a tag for identification of the labeled region is thus attractive. One such application is to use a stable isotope for mass analysis, stable isotopes being easier to handle than radioisotopes. Recent progress in mass spectrometry has opened the way to identify labeled components through MS/MS experiments (Figure 14).[33] Stable isotopes are very useful for tags as exogenous components of labeled components in mass spectrometry, because the altered ratio mixture of unlabeled and labeled reagents affords the corresponding ratio of labeled biomolecules with different mass numbers. Synthesis of the stable-isotope-labeled TPD is easier than with radioisotopes, because there are fewer scale limitations for the synthesis and treatment is easier. Several stable isotope introduction methods with "post-functional" synthetic methods have been reported. In particular, deuterium-labeled TPD derivatives are easier to prepare than their tritiated counterparts. The deuterium atom is introduced into the ligand skeleton or a spacer moiety is used. Introduction of deuterium into the diazirine moiety has recently been examined; examples have included α-hydrogen displacement with LDA/D_2O (compound **38**),[34] specific hydrogen replacement in a C–I bond (**39**),[24] reduction of an aldehyde or acyl halide with $NaBH_4$ (**40**),[3,35] regiospecific hydrogenation of a 1,1-dibromoalkene (**41**, **42**),[26] reduction of the aryl carbonyl **43** to provide methylene compound **44**,[36] and hydrogenation of a C=C bond under deuterium in the presence of Wilkinson's catalyst to afford **45**.[25] These compounds were synthesized from unlabeled (cold) diazirine compounds and commercially available deuterated reagents.

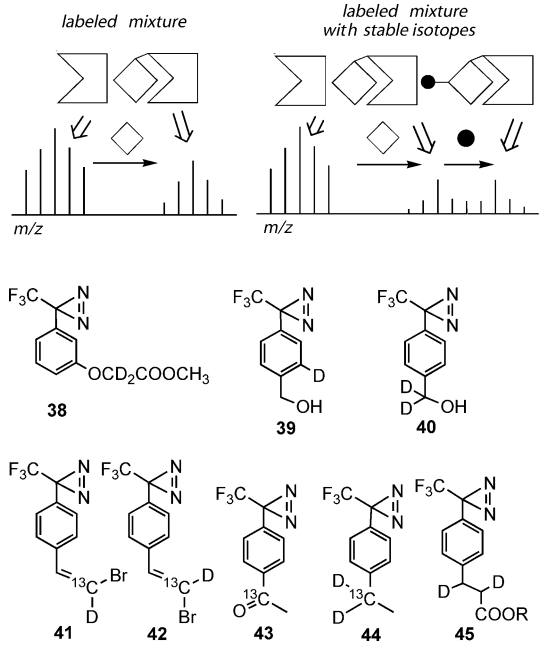

Figure 14. Combinations of photoaffinity labeling and stable isotopes with mass analysis and stable isotope-containing TPD derivatives.

iii) Avidin–Biotin System

Specific interaction between avidin and biotin has been well studied. Avidin, which consists of tetrameric subunits, recognizes the small molecule biotin with very strong affinity ($K_d = 10^{-15}$ M).[37] Other known biotin-specific biomolecules are streptavidin, anti-biotin antibody, and monomeric avidin. Streptavidin[38] is purified from *Streptmyces avidinii* and has the same affinity for biotin as avidin, but the pI of the protein is between 5–6, whereas the pI of avidin is almost basic (pI 10). Furthermore, streptavidin is nonglycosylated. These differences suppress unspecific binding of biotinylated compounds to streptavidin in relation to avidin. Anti-biotin antibody[39] also shows that biotin is less sensitive than (strept)avidin ($K_d = 10^{-9}$ M). It is useful that endogenous (strept)avidin interferes in the detection of biotin compounds. Monomeric avidin, which is only prepared on a solid support, has weaker affinity than avidin ($K_d = 10^{-9}$ M)[26] and dissociation of the avidin–biotin complex is easier than that of the tetrameric form (2 mM biotin or glycine buffer pH 2.0). This specific interaction has been combined with photoaffinity labeling (photoaffinity biotinylation). The combination was first developed with a peptide ligand, because photophores and biotin can be introduced separately at lysine α- and ε-amino groups,[40] but not all ligands have many modification sites in the ligand skeleton. One solution is the synthesis of a biotinylated photophore. Synthesis methods based on "post-functionalization" have enabled us to produce many biotinylated diazirinyl derivatives.[20] The avidin–biotin complex was detected by chemiluminescence detection with the same sensitivity as achieved by radioisotope analysis. The *N*-acetylglucosamine-asparagine skeleton, which is a substrate for β-1,4-galactosyltransferase, was modified with biotinylated diazirinyl photophore and the binding site in the protein was revealed for the first time (Figure 3, Figure 15).[5,41]

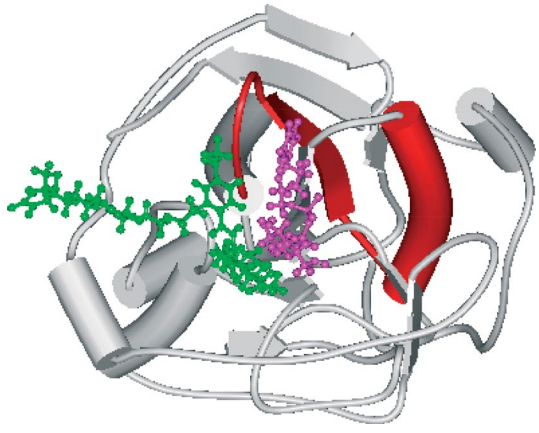

Figure 15. A docking model of β-1,4-GalT.

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Avidin–biotin interaction is very strong, and formation of the complex is very fast and reliable, but it is too difficult to dissociate the biotin molecule from the complex as a native form. Very harsh conditions are needed for the biomolecules (70% HCOOH at room temp., autoclave or 2% SDS/8 M urea with boiling) and the recovery yield of the biotinylated biomolecule is not quantitative. Since biotin–(strept)avidin binding is essentially irreversible, several approaches to achieve the efficient recovery of biotinylated products from a (strept)avidin-immobilized matrix have been investigated.

In one such approach, modified avidin, as described above, was used. Another introduced chemically cleavable tags between the photophore and biotin for isolation of photolabeled components from the avidin–biotin complex (Figure 16). Diol and disulfide moieties as in **46** and **47**[42] are well suited for this purpose with isolation with thiol and periodate, respectively, although these moieties (disulfide bond and sugar moiety) are also present in biomolecules, so it is not a specific method for labeled biomolecules. Recently, it was reported that the nitrophenyl system, as in **48**,[43] and the sulfonamide system, as in **49**,[44] are suitable for the purpose (Figure 17). These moieties are less abundant in biomolecules and the cleaving conditions are very mild in almost all biomolecules. Photoaffinity labeling with sulfonamide derivatives for several lectin systems demonstrated the utility of the moiety for isolation of labeled components.

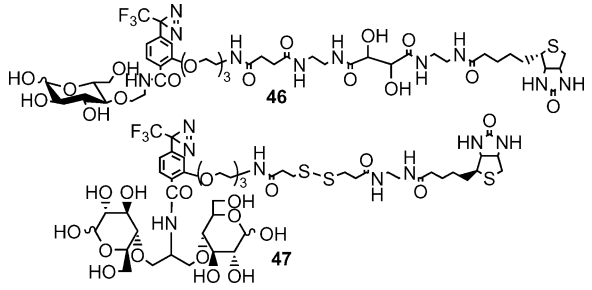

Figure 16. Biotinylated TPD analogues between TPD and biotin that are chemically cleavable by periodinate (**46**) and disulfide reduction (**47**).

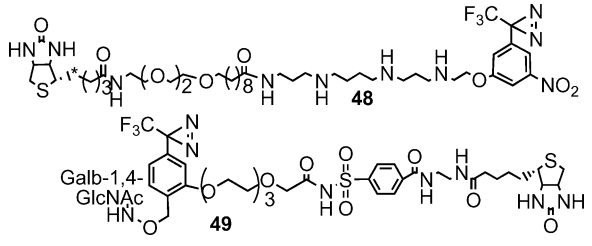

Figure 17. Biotinylated TPD analogues between TPD and biotin that are chemically cleavable by photolysis (**48**) and nucleophilic cleavage (**49**).

The final possibility is to utilize site-specific digestion enzymes for the ligand skeleton.[45] The protected amino acid residue was inserted between diazirine and biotin, was easily deprotected, and then acted as a substrate for digestion enzymes (cf. **50**, **51**; Figure 18). Folding biomolecules were not digested under these conditions, although the introduced amino acid also became a substrate when the protecting groups were removed. Digestion was regulated through methyl esters, a basic protecting group in organic chemistry. This combination was utilized not only in protein mixtures but also in digested mixtures. Use of the combination of the glutamic acid γ-methyl ester and V8 protease revealed that effective retrieval of labeled components is possible. Labeled biocomponents are first enzymatically digested and then deprotected, followed by redigestion to cleave the avidin biotin complex to isolate the labeled peptides. Furthermore, the combination of linker amino acid and digested enzyme had many patterns to facilitate handling of the labeled components.

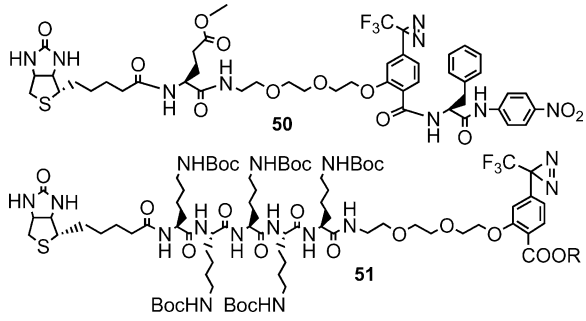

Figure 18. Enzymatically cleavable TPD analogues.

iv) Post-Modification of Labeled Components

Although photoaffinity biotinylation was very useful for identifying the labeled region, the large modification with the ligand skeleton sometimes causes decreased affinity in the synthetic compounds. Post-modification of labeled components solved this problem. Reduction of a disulfide bond followed by thiol modification was utilized for this purpose in carbohydrate-related proteins.[46] The post-functional concept has also been applied to the activity-based protein profiling (ABPP) approach.[47] Hosoya et al. reported that use of an alkyl azide such as **52**[29] with Staudinger ligation (triphenylphosphane derivatives) and of click chemistry (alkyne derivatives such as **53**[48]) in the aqueous phase were applicable to photoaffinity labeling. The post-introduction of a tag could also be applied to avidin–biotin systems, but the use of less of the labeled component hampered effective post-biotinylation of the labeled protein (Figure 19).[49]

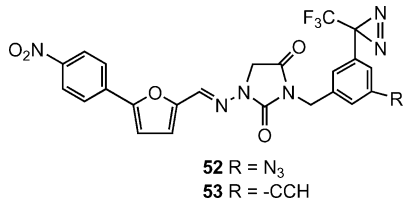

Figure 19. TPD derivatives for Staudinger reaction (**52**) and click chemistry (**53**).

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d) Application in Protein Network Analysis

In the previous section, photoaffinity biotinylation proved useful at the purified biomolecular level. Photoaffinity labeling is useful for targeting of biomolecules in complex systems because it is based on affinity to the target molecule. This section describes attempts to apply complex systems.

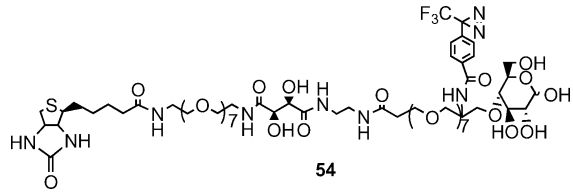
i) Expressed Protein Mixtures

Contaminants of the target biomolecule were very high in expressed protein mixtures. Photoaffinity labeling to target biomolecules was carried out essentially as specified, although without purification of the target biomolecules. Photoaffinity biotinylation was reported for a crude extract of expressed human β-1,4-galactosyltransferase. The results indicated that the ligand was only recognized by the active protein, and no unspecific labeling (interaction) of other proteins was observed. It should be pointed out that photolabeling for the expressed protein sometimes provided no further purification of the expressed protein mixture. The same results were reported for photolabeling of thermolysin, which was mixed with yeast extract (containing other proteins), and then subjected to photolabeling with fluorophore-containing TPD. The results indicated that TPD was incorporated into thermolysin. On the other hand, a benzophenone derivative was not recognized in the yeast-extracted mixture.[50]

ii) Photoaffinity Labeling (Biotinylation) of the Glucose Transporter on Cell Surfaces

We developed TPD photoaffinity biotinylation of the glucose transporter (GLUT) as a typical experiment for living cells. GLUT proteins are of particular importance as pharmacological targets, and many GLUTs are membrane proteins for maintaining homeostasis. Among GLUTs, GLUT4 protein is of particular importance as it is present only in insulin-responsive tissues.[51] In the basal state, GLUT4 is sequestered into an intracellular reservoir membrane compartment. The exposure of GLUT4 at the cell surface is regulated by insulin signaling, which initiates a signaling cascade that ultimately results in stimulation of the exocytosis of GLUT4-containing vesicles and fusion of the vesicles with the plasma membrane.[52] This process is defective in type II diabetes, and methods for rapid monitoring of the extent of GLUT4 translocation between membrane compartments are therefore needed. Previous studies revealed that the C-4 hydroxy position in hexoses tolerate the introduction of the bulky substituent to provide substrate GLUTs. Photoaffinity labeling with C-4-modified hexose derivatives is particularly applicable to membrane glucose transporters (GLUTs) that expose short exofacial loops at the outer surface of the cell that are difficult to label by conventional biochemical approaches. Bis-mannose derivatives in which aryl azide[53] or benzophenone groups[54] have been introduced have been reported. The disadvantages of aryl azides and benzophenone are a result of the same problem as discussed in the previous sections. In contrast, TPD derivatives have been found to generate highly reactive carbene smoothly and specifically with short irradiation times.[55] The properties suggested that TPD photolabeling should be applicable to investigate the cell trafficking assay for GLUT4[56] and other GLUTs.[57]

Several radioisotope hexose photoaffinity probes were used in the first stages, but their handling and synthesis difficulties prevented further investigation. The combination avidin–biotin system is an alternative, but photoaffinity biotinylation with diazirine photophores for GLUTs on intact cells encountered detection problems. Several biotinylated diazirinyl compounds were tested for GLUT4 labeling on the cell surface. It seems that the cell outer surface components (glycolipids, glycoprotein, etc.) inhibit the access of avidin to introduced biotin on the membrane. Avidin is able to access membrane-bound biotin, and at least 60 atoms between the photophore and diazirine **54** are needed (Figure 20).[58] Photoaffinity biotinylation of GLUT1 on human erythrocytes afforded the same results, compounds containing shorter linkers between photophore and biotin not recognizing (strept)avidin. Photoaffinity labeling of biocomponents on the cell surface represents an attractive field of in vivo functional analysis.



54

Figure 20. TPD analogue (**54**) for photoaffinity labeling of glucose transporter on the cell surface.

Conclusions

Photoaffinity labeling techniques can be applied not only to biomolecules and ligand interaction but also to a new generation of solid-phase chemistry.[59] For this purpose, the fast and easy preparation of TPD derivatives is fundamental. The TPD skeleton was thought to be very labile under many sets of synthesis conditions until the early 1990s, but the unexpected stability of the TPD three-membered ring against various synthesis conditions has encouraged researchers to extend the application of TPD. Progress in diazirine-based photoaffinity labeling in intact systems is ongoing day to day and should reveal detailed mechanisms of bioactivity in the near future.

Acknowledgments

This research was partially supported by a Ministry of Education, Science, Sports and Culture Grant-in-Aid for Scientific Research on a Priority Area, 18032007, for Scientific Research (C), 19510210

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(M. H.), and (B) 18390036 (Y. H). M. H. also thanks the Fugaku Foundation and Research for Promoting Technological Seeds for financial support for the study. Y. H. thanks the Japan Science and Technology Agency (JST), Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER).

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Received: November 12, 2007
Published Online: March 4, 2008

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