Recent progress in diazirine based photoaffinity labeling

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Abstract

Based on the recent development of molecular biology, the investigation of biofunctional machinery at their ligand accepting interface has become one of the challenging and important subject of post-genome field. The technique of photoaffinity labeling has become increasingly appreciated as a powerful methodology for the purpose. This micro review focused synthesis of trifluoromethylphenyldiazirine, which is one of the most promising photophore, and its application to the biomolecules.

Biographical Sketches

Makoto Hashimoto was born in Hokkaido, Japan in 1966. He received both his B.S. (1989) and Ph.D. (1995) from Hokkaido University and conducted his postdoctoral research at Toyama Medical and Pharmaceutical University (1995–1997) as a Research Fellow of the Japan Society for the Promotion of Science, and at the University of Bath, UK (1999–2000) as a Research Officer. In 1997, he joined the bioorganic laboratory at Obihiro University of Agriculture and Veterinary Medicine where he was appointed Associate Professor in 2003. His research is concentrated on defining and understanding the mechanisms of bioactive compounds with bioorganic methods.

Yasumaru Hatanaka received his B.S. (1971) and M.S. (1973) from Kanazawa University, and his Ph.D. (1976) from Hokkaido University. After working as a JSPS Postdoctoral Fellow (1976–1977), he joined the Faculty of Pharmaceutical Sciences, Hokkaido University as a Research Associate (1977–1990). During this period, he also worked as a Postdoctoral Fellow at Rockefeller University (1984–1986). He became an Assistant Professor in 1990 and then moved to the Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, as an Associate Professor in

1992. Since 2000, he became to a Full Professor in the Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University. Since 2005, the University name was renewed as University of Toyama and he has been the Dean of the Graduate School of Medicine and Pharmaceutical Sciences. He received the PSJ (Pharmaceutical Society of Japan) Award for Young Scientists (1994). His research interests are in the areas of chemical biology, medicinal chemistry, and proteomics.

Introduction

Elucidation of protein functions based on the structure-activity relationship is one of the greatest interests for scientists to reveal the mechanisms of homeostasis functions in life. 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 by deleting or mutating the native amino acid alignment of proteins. When the target protein can be expressed in a large quantity, 3D structural determinations which include NMR spectroscopy and homology modeling are a powerful and direct approach for analyzing the three-dimensional structure 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 structure because these analyses are based on the affinity between the ligand and target protein (Figure 1). Affinity-based chemical modification introduces a useful tag for analyzing the target protein. For example, single molecular imaging with fluorophore[1] visualizes target biomolecules in complex systems for imaging the localization of biomolecules and processing the flow of bioactive compounds in cell compartments. For specifically attaching the desired tag on the target protein, affinity labeling[2] has a limited role because the method requires the presence of nucleophilic residues near to a ligand binding site for preventing nonspecific introduction of the tag to a site different from the binding site or on other co-existing biomolecules. But recent progress of molecular biology prompted site-directed labeling using cysteine mutants to overcome this problem. [3]

Photoaffinity labeling[4] largely increased the capability of specific tagging. Photochemically generated highly reactive spices introduce covalent bonds between ligand and protein in a

nonselective manner and any amino acid in the binding site can be tagged (Figure 2).

One of the successful applications of photoaffinity labeling is the identification of target biomolecules in crude extracts using 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 was destroyed by the denaturation condition, which allows further sophistication in detailed structural analysis, and the detection of tagged components may reflect the status of conformational changes of target molecules. Our previous study on β-1,4-galactosyltransferase demonstrated that photoaffinity labeling can reveal conformational change during the enzymatic reaction. The enzyme has two substrates, UDP-galactose **1** 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 in the presence of UMP, which is a UDP-Gal analogue at 37 °C. The results indicated that the two substrates should be orientated near position in the active site to promote conformational change of the enzyme structure (Figure 3).[5] Recently it was reported dynamic structural investigations on the torpedo nicotinic acetylcholine receptor was performd by time-resolved photoaffinity labeling.[6]

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 low photo-incorporation yield hampered the identification of photolabeled components.[4, 7] There are many choices for photoaffinity labeling experiments, choice and synthesis of the photophore, photolysis condition, and choice tags for the identification of biocomponents.

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

This microreview focuses on the fundamentals of photoaffinity labeling experiments and the recent

development of photoaffinity labeling with (3-trifluoromethyl)phenyl diazirine, one of the most promising photophores, and its applications for the effective analysis of ligand-biomolecules interactions.

a) Photophore synthesis

i) Advantage of diazirine photophore

It is important which photophores are used for effective photoaffinity labeling (Figure 4). Typically, aryl azide, benzophenone and aryl diazirine are used. Aryl azide is photoactivated at lit lower 300 nm, which sometimes causes damage to biomolecules, and generates nitrene[8] as an active species, which is sometimes rearranged to keteimine as an undesired side product.[9]

Benzophenone[10] is photoreactivated with light over 350 nm and generates reactive triplet states of carbonyl, which regenerates ground-state carbonyl compounds and is re-usable for photolabeling, although the photophore sometimes needs long photoirradiation for labeling.

Aryldiazirine[11] is also photoreactivated with light over 350 nm and generates carbene, which is a higher reactive species than other photophores, rapidly forming cross-links to biomolecules with short photoirradiation. It was reported that the photolysis of diazirine causes diazo isomerization, which is an undesired intermediate in photoaffinity labeling. Diazo isomerization is suppressed by introducing a trifluoromethyl group to a diazirinyl three-membered ring.[12] Comparative irradiation studies of these three photophores in living cells suggested that irradiation for the generation of active species from azide and benzophenone caused cell death during photolysis because a long irradiation time is needed to incorporate the cell membrane surface. On the other hand, a carbene precursor, (3-trifluoromethyl)phenyldiazirine (TPD) did not promote cell death to generate active species, but other photophores are sometimes utilized in photoaffinity experiments in vitro (i.e. γ-secretase).[13] Although these results indicated that photoaffinity labeling with TPD has the most promising character to investigate chase of labeled components in the cell, the applications are not

as many as with other photophores, because synthetic construction of TPD skeleton is not easier than other photophor.

ii) Efficient synthesis of TPD

Synthesis of the TPD three-membered ring required at least five steps from the corresponding aryl halide derivatives (Figure 5A). A tether should be introduced on the benzene ring to connect the ligand skeleton. Previous preparations of TPD derivatives had a pre-installed suitable tether before construction of the diazirine moiety, but it seems that the diazirinyl three-membered ring is not stable for many organic reactions. The repeated construction of a diazirine moiety for each derivative is a drawback for applying the photophore to photoaffinity labeling. Our breakthrough work on "post-functional" of diazirinyl compounds[14] revealed that the three-membered ring was stable under many organic reaction conditions. Although the trifluoromethyl diazirinyl moiety is categorized as an alkyl substituent, polarization of the quaternary carbon atom is slightly positively charged, so the moiety was less activated for electrophilic aromatic substitution than unsubstituted. [15] First, we selected *m*-methoxy substituted TPD **5** as the mother skeleton, because 1) the methoxy group was strongly activated for electrophilic aromatic substitution, 2) orientation of the substitution preferred the *o*-position against the methoxy group, because the *p*-position is sterically hindered by trifluoromethyl diazirinyl moiety, 3) de-methylated reaction for *m*-methoxy TPD was easier than for *p*-methoxy TPD, and the re-alkoxy reaction of the demethoxy group was utilized as the tag introduction (Figure 5B). Compound **5** introduced a nitro group easily 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**. Iodinated derivative is a useful compound for radioisotope introduction. Iodination for compound **5** was achieved by 1) via thallic derivatives, 2) via alkyl tin derivatives or 3) with hypervalent iodine reagent. Friedel-Crafts alkylation with dichloromethyl methyl ether for **7**, followed by hydrolysis afforded aldehyde derivative **8**[16], which

was previously reported as an aldehyde group preinstalled before diazirine construction.[17] Aldehyde **8** was easily converted to alcohol **9** by $NaBH_4$-methanol, carboxylic acid **10** by manganese and cinnamic acid derivatives **11** by Wittig reaction with stable ylide in the usual manner.[16] Wittig reactions with unstable ylide for **8** had very low yield because of strong basic conditions.[18] Friedel-Crafts acylations are also widely utilized for compound **5**. These reductions were one of the most incompatible reactions for TPD derivatives in the presence of an easily reducible nitrogen-nitrogen double bond. We tried to reduce the aryl carbonyl group to methylene 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 study, reduction with triethylsilane - trifluoroacetic acid was utilized for alkylphenone-type TPD derivatives **12** and the reaction was applied for the synthesis of photoreactive fatty acid analogue **13**.[19]

The *m*-methoxy group was easily hydrolyzed to phenolic derivative **14** with BBr_3, compared with *p*-methoxy substituent.[14a] Compound **14** is used as starting material to prepare re-methylation **15** with radiolabeled methyl iodide, enzymatic or chemically iodination **16**[14] and O-alkylations **17**, **18**, [20] which contains biotinylation described later (Figure 7).

Although the methoxy group was helpful in the aromatic substituent reactions, it sometimes caused less activity of the targeted biomolecules. We attempted post-functional derivatization for unsubstituted TPD **19** under various conditions (Figure 8). As mentioned previously, diazirinyl moiety acts as a more electron-withdrawing group than unsubstituted group, 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 sufficient 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. A stronger acid, trifluoromethanesulfonic acid (TfOH) with SbF_5 or $TiCl_4$, without solvent can easily 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]

Selective reduction of the carbon-carbon double bond to alkane in the TPD derivative is problematic. TPD nitrogen-nitrogen double bond is destroyed by heterogeneous catalysis, Pd/C, under a hydrogen atmosphere for over an hour.[24] On the other hand, hydrogenation with Wilkinson's catalyst for **22**, which is 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, then introducing substituent groups to be used as tethers or tags. These "post-functional" derivatives from mother compounds have been performed by not only our research group[26] but also other researchers.[27]

In these reactions, there are many incompatible reactions with other photophores, azide and benzophenone. These shorter methods for prepare photophores prompted the routine use of the diazirinyl photophore for photoaffinity labeling experiments.

b) Photoreaction

i) Ligand concentration

One of the best conditions for the photoaffinity labeling experiment is the addition of stoichiometric amounts of labeled reagents against a biomolecule, because the technique involves the binding site with labeled reagents, and excess reagents sometimes cause serious unspecific labeling at expected position. Figure 9 shows the photoaffinity labeling of erythrocytes with different amounts of biotinylated photoligand **24**. Excess photoactive ligand caused less inhibition of photolabeling with 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 (Km, Ki, Kd etc) are already known, 10 times higher against the affinity parameters to complete the ligand - biomolecule complex.[5a]

ii) Side reaction

It was reported 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 carbene with a shorter wavelength (305 nm)[29] or longer irradiation with a black light.[12] These photolysis experiments were preformed for ligand concentrations over 10 mM. On the other hand, actual photolabeling for biomolecules was performed less than 1 mM. ^{19}F- NMR studies of photodecomposition for compound **25** at 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 10B). Serious diazo isomerization was observed at 10 mM, but smooth photolysis was observed after re-irradiation after the isomerized sample was diluted to less than 1 mM. These results were consistent with actual photolabeling, L-amino acid oxidase with 0.01 mM of **28** and chymotrypsin with 0.2 mM of **29**. There were no differences between irradiation by 350 nm light alone (Figure 11B lanes 1 and 3) and one by 350 nm followed by 302 nm (Figure 11B lanes 2 and 4).[30]

iii) Labeling efficiency

Even though the photolabeling condition was set up as indicated above, photolabeling efficiency, which generates a covalent bond between photophores and biomolecules, was always less than 10%. This depends on many factors, orientation of photophores, transmittance of the photolabeling mixture, quenching generated carbene by water, and affinity of the ligand, etc, but labeling efficiency is less than 10% of the used biomolecules. It was sometimes observed as near ~30% efficiency for high affinity biomolecules (i.e. ion channels, receptors Kd $<10^{-9}$ M).[31] This is no

problem for the detection of labeled components, because highly sensitive methods (radioisotope, etc) were applied to the detection, but this low labeling efficiency hampered the identification of the labeled portion in biomolecules. Several approaches were examined to solve these difficulties and are summarized in the next section.

c) Progress in the 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, e] Here we briefly describe the fundamentals of several concepts.

i) Immunological methods

It is well known that antigen-antibody interactions have very specific recognition, and generation of the antibody to biomolecules has been well studied. Recent developments suggest the preparation of an antibody against part of the target biomolecules (i.e. selected peptide sequence). Specific antibodies, which recognized part of the target biomolecules, were used for the photolabeled mixture in which 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 can 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]

ii) Chemical tags in the mass spectrometry

Radioisotopes have been utilized to detect labeled components for over three decades, because high radioactivity helps to detect photolabeled components, but the synthesis of radioactive TPD derivatives is not easy, because limited amounts of radiolabeled reagents are used for synthesis. One of the methodologies introduced radiolabeled iodine into the benzene ring of TPD compound **31**, which was synthesized from the corresponding amino derivatives with diazotization followed by iodination. Iodine was introduced by NaI - chloramine T or KI-hydrogen peroxide for phenolic TPD derivatives **32**, **33**. Less activated TPD derivatives for electrophilic substitutions were used to exchange between organic thallium[14a] or tin[27] and molecular iodine to afford **34**, and 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 by the exchange of cold iodine and tritium gas in the presence of Pd/C within one hour in **35**. Phenoxy TPD **14** was easily introduced into both tritium **36** and ^{14}C **37** by de- methylation and re-methylation with radiolabeled methyl iodide.[14a] The limitation of specific (radio)activities of tritium and ^{14}C reagents is sometimes problematic. Furthermore, it is frequently observed that the yields of radioisotope synthesis are lower than the corresponding cold reagents, and the handling and storage of radiolabeled compounds are greatly hampered. The radiolabeled–free synthesis of compounds that contain a tag for identification of the labeled region is promoted. One of the applications is to use the stable isotope for mass analysis. Stable isotopes are handled easier than radioisotopes. Recent progress of mass spectrometry has opened the way to identify labeled components with 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 less scale limitations for the synthesis and treatment is easier. Several stable isotope introduction methods have been reported with "post-functional" synthetic methods. In particular, deuterium-labeled TPD derivatives were easier to prepare than with tritium. The deuterium atom is introduced into the ligand skeleton or a spacer moiety is reported. Recently, deuterium introduced on the diazirine moiety was examined, for example, α-hydrogen displacement with LDA-D_2O **38**,[34] specific hydrogen replacement of C-I bond **39**,[24] reduction of aldehyde or acyl halide with $NaBH_4$ **40**,[335] regiospecific hydrogenation 1,1-dibromo alkene **41, 42**,[26] reduction of the aryl carbonyl **43** to methylene **44**[36] and hydrogenation of the C=C bond under a deuterium atmosphere with Wilkinson's catalyst to afford **45**.[25] These compounds were synthesized from unlabeled (cold) diazirine compounds and commercially available deuterated reagents.

iii) Avidin-biotin system

Specific interaction between avidin and biotin has been well studied. Avidin, which consists from tetrameric subunits, recognizes small molecule biotin with very strong affinity ($K_d = 10^{-15}$ M).[37] Other biotin-specific recognized 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 non-glycosylated. These differences suppress the unspecific binding of biotinylated compounds to Streptavidin rather than 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 detecting biotin compounds. Monomeric avidin, which is only prepared on a solid support, has less affinity than avidin ($K_d = 10^{-9}$ M)[26] and dissociation of the avidin-biotin complex is easier than the tetrameric form (2 mM biotin or glycine buffer pH 2.0). This specific interaction

was combined with photoaffinity labeling (photoaffinity biotinylation). The combination was first developed to 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 resolution is the synthesis of biotinylated photophore. Synthesis methods based on "post-functionalization" enable us to produce many biotinylated diazirinyl derivatives.[20] The avidin-biotin complex was detected to the same degree as radioisotope analysis by chemiluminescence detection. The *N*-acetyl glucosamine-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 (Figures 3, 15).[5, 41]

Avidin-biotin interaction is very strong, and formation of the complex is very fast and rigid, 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 rt, autoclave or 2% SDS - 8M urea with boiling) and the recovery yield of the biotinylated biomolecule is not quantitative. Since biotin-(strept)avidin binding is essentially irreversible, several approaches have been investigated to achieve the efficient recovery of biotinylated products from (strept)avidin-immobilized matrix.

Several approaches were examined for this problem. In one, modified avidin, which is described above, was used. Another introduced the chemical cleavable tags between the photophore and biotin to isolate photolabeled components from the avidin-biotin complex (Figure 16). Diol **46** and disuldide **47** moieties[42] are well used for this purpose with isolation with thiol and periodate, respectively, however these moieties are also present in biomolecules (disulfide bond and sugar moiety), so it is not a specific method for labeled biomolecules. Recently, it was reported that nitrophenyl **48**[43] and sulfonamide moiety **49**[44] were suitable for the purpose (Figure 17). These moieties are less abundant in biomolecules and the cleavable condition is very mild in almost all biomolecules. Photoaffinity labeling with sulfonamide derivatives for several lectin demonstrated

the utility of the moiety to isolate labeled components.

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, and was easily deprotected, and then acted as a substrate for digestion enzymes **50, 51** (Figure 18). Folding biomolecules were not digested under this condition, although the introduced amino acid also became a substrate when the protecting groups were removed. Digestion was regulated by a methyl ester, which is a basic protecting group in organic chemistry. This combination was utilized in not only protein mixtures but also digested mixtures. The combination of the glutamic acid γ-methyl ester and V8 protease revealed that effective retrieval of labeled components is possible. For labeled biocomponents, it is first enzymatically digested, and then deprotected, followed by re-digestion 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.

Although photoaffinity biotinylation was very useful to identify the labeled region, the large modification with the ligand skeleton sometimes causes decreased affinity of the synthetic compounds. Post modification of labeled components solved this problem.

iv) post-modification of labeled components

Although photoaffinity biotinylation was very useful to identify the labeled region, the large modification with the ligand skeleton sometimes causes decreased affinity of the synthetic compounds. Post modification of labeled components solved this problem. Reduction of disulfide bond followed by thiol modification was utilized to carbohydrate related proteins for this purpose.[46] The post-functional concept was also applied to activity-based protein profiling (ABPP) approach.[47] Hosoya et al. reported that alkyl azide with Staudinger ligation (triphenyl phosphine derivatives) **52**[29] and click chemistry (alkyne derivatives) **53**[46] in the aqueous phase were applied to photoaffinity labeling. The post-introduction of tag will also be applied to avidin-biotin systems,

but less of the labeled component hampered effective post-biotinylation of the labeled protein (Figure 19).[47]

d) Application in the protein network analysis

In the previous section, photoaffinity biotinylation was useful at the purified biomolecular level. Photoaffinity labeling is useful to target biomolecules in complex systems because it is based on affinity to the target molecule. In this section, attempts to apply the complex system are described.

i) Expressed protein mixtures

Contaminants of the target biomolecule were very high in the expressed protein mixtures. Photoaffinity labeling to target biomolecules almost proceeded 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 unspecific labeling (interaction) of other proteins was not observed. It should be indicated 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, benzophenone derivative was not recognized in the yeast-extracted mixture.[48]

ii) Photoaffinity labeling (biotinylation) of glucose transporter on the cell surface

We developed the TPD photoaffinity biotinylation to 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.[49] 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.[50] This process is defective in Type II diabetes and, therefore, methods for rapid monitoring of the extent of GLUT4 translocation between membrane compartments are needed. The previous studies revealed that C-4 hydroxyl position of hexose tolerated the introduction of the bulky substituent as substrate GLUTs. Photoaffinity labeling with C-4 modified hexose derivatives particularly applies to membrane glucose transporters (GLUTs) which expose short exofacial loops at the outer surface of the cell which are difficult to label with conventional biochemical approaches. Bis-mannose derivatives have been reported in which aryl azide[51] or benzophenone groups[52] have been introduced. The disadvantages of aryl azides and benzophenone faced the same problem, which was discussed in the previous section. By contrast, TPD derivatives have been found to generate highly reactive carbene smoothly and specifically with short irradiation times.[53] The properties suggested TPD photolabeling should be applied to investigate the cell trafficking assay for GLUT4[54] and other GLUTs.[55]

Several radioisotope hexose photoaffinity probes were used in the first stage, but their handling and synthesis difficulties prevented further investigation. Combination avidin-biotin system is an alternative, but photoaffinity biotinylation with diazirine photophores for GLUTs on intact cells faced 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 are needed between the photophore and diazirine **54** (Figure 20).[56] Photoaffinity biotinylation of GLUT1 on human erythrocyte afforded the same results, compounds contained shorter linkers between photophore and biotin did not

recognized with (strept)avidin. Photoaffinity labeling of biocomponents on the cell surface will be an attractive study to analyze the mature role in complex systems.

Conclusion

Photoaffinity labeling techniques will be developed as applications for not only biomolecules and ligand interaction but also a new generation of solid phase chemistry.[57] For this purpose, the rapid preparation of TPD derivatives is fundamental. The TPD skeleton was thought to very labile in many synthesis conditions until the early 90's, but unexpected stability of the TPD three- membered ring against various synthesis conditions encouraged researchers to extend the application of TPD. The progress of diazirine-based photoaffinity labeling in intact systems is ongoing by day and will reveal the detailed mechanisms of bioactivity in the near future.

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Figure legends

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

Figure 2 Schematic diagram of photoaffinity labeling.

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

Figure 4 Photochemical reactions of major photophore for photoaffinity labeling.

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

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) $(nBu)_4NMnO_4$, vii) $Ph_3CCHCOOR$, viii) RCOCl, $AlCl_3$, ix) Et_3SiH, TFA.

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) $Br(CH_2)_{10}COOCH_3$, then aqueous NaOH.

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

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

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 B).

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 minutes, and also with black light for 10 minutes followed by light at 302 nm for 8 minutes (lanes 2 and 4). Photolabeled proteins were subjected to SDS-PAGE followed by chemiluminescence detection for quantitative densitometry analysis.

Figure 12 TPD derivatives of galactosylceramide

Figure 13 Radioactive TPD derivatives.

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

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

Figure 16 Biotinylated TPD analogues chemical cleavable between TPD and biotin by periodinate (**46**) and thiol (**47**).

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

Figure 18 Enzyme cleavable TPD analogues.

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

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

Figure 1

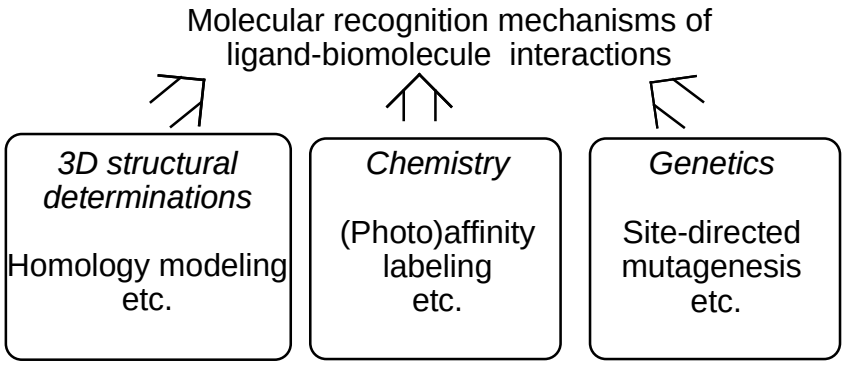


Figure2

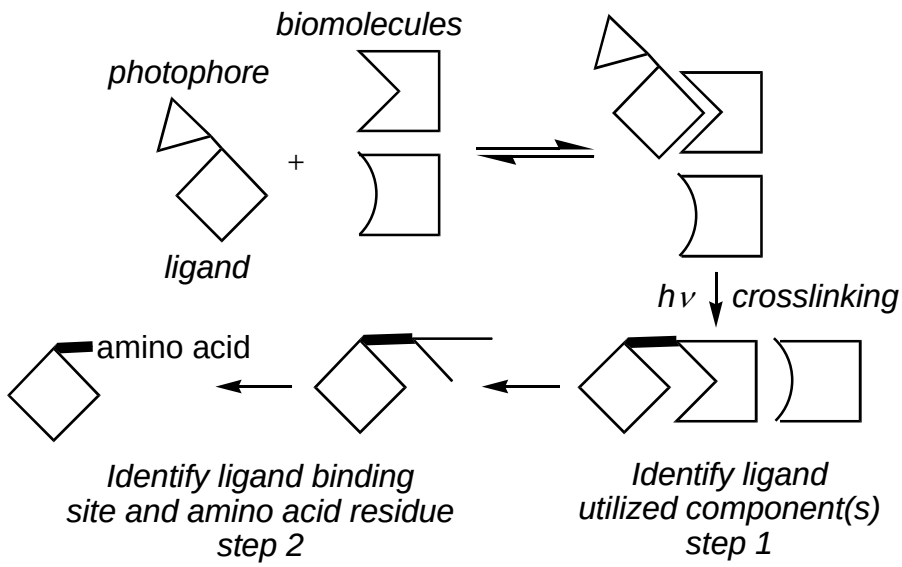


Figure 3

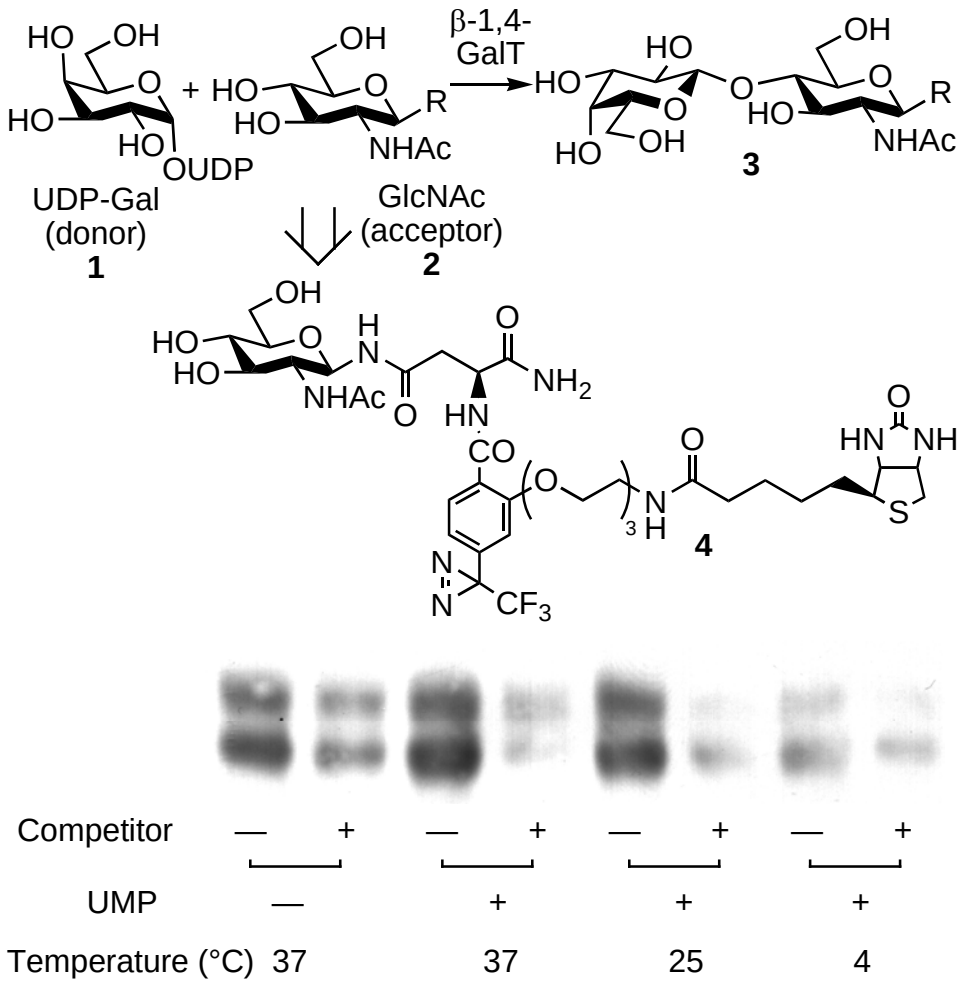

β-1,4-
GalT
UDP-Gal
(donor)
1
GlcNAc
(acceptor)
2
3
4
Competitor — + — + — + — +
UMP — + + +
Temperature (°C) 37 37 25 4

Figure 4

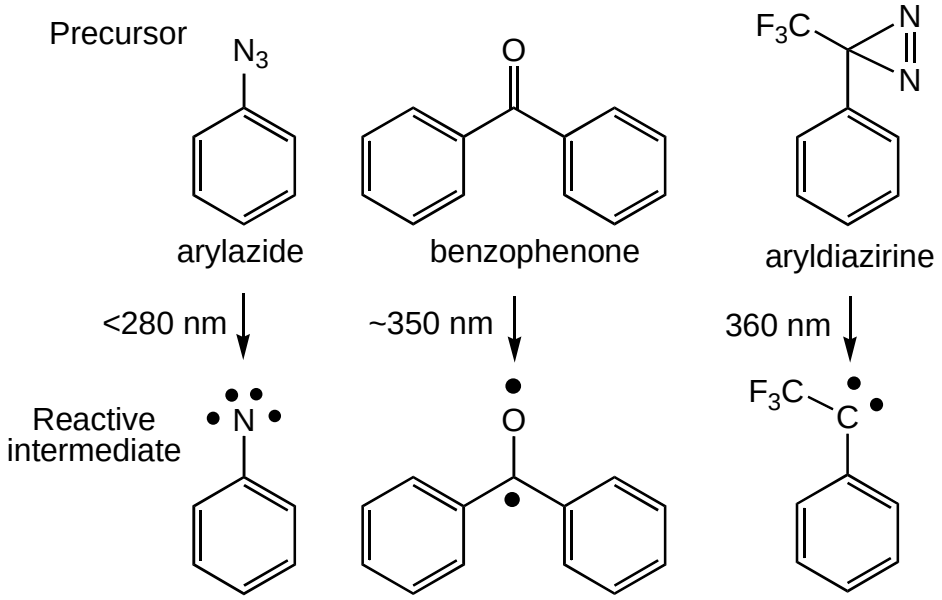
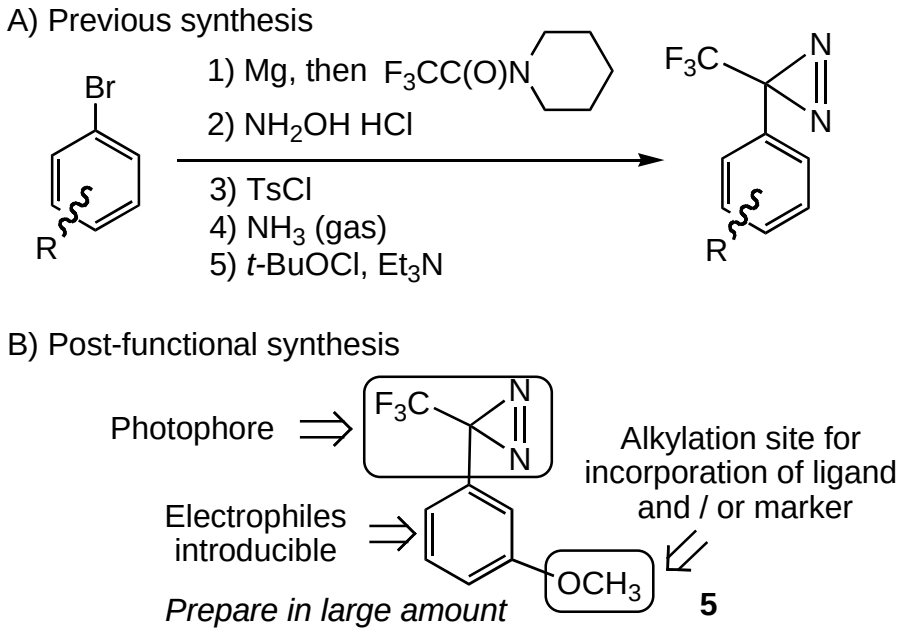


Figure 5



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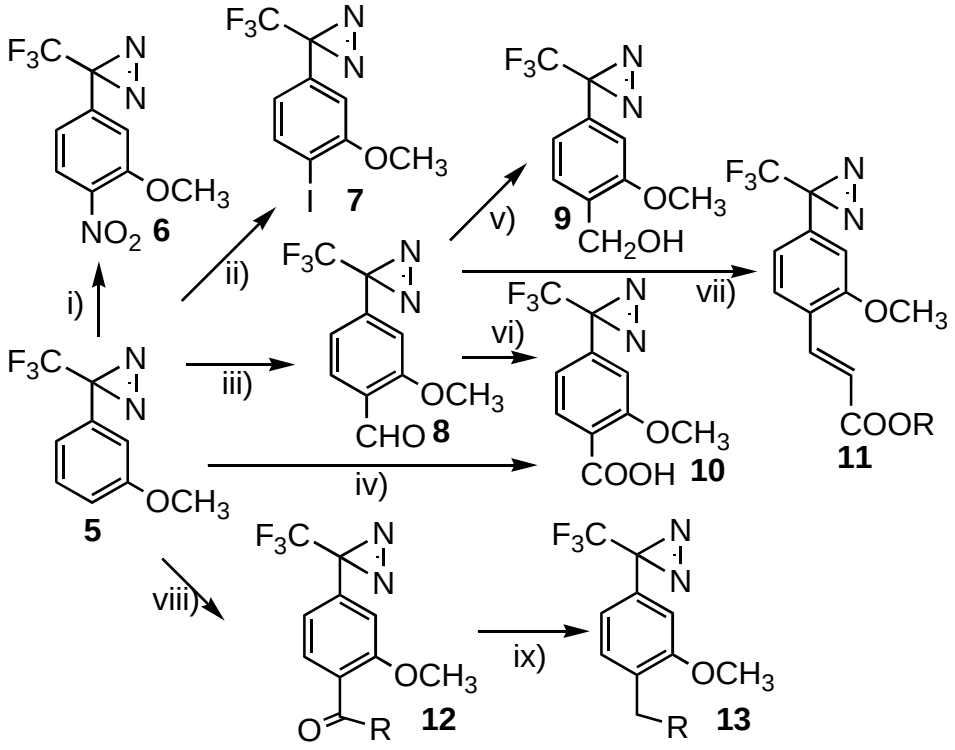
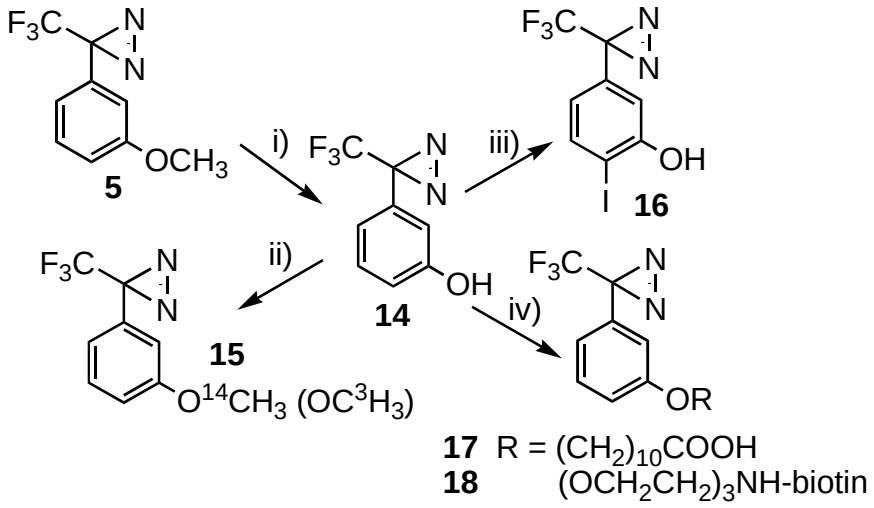


Figure 7



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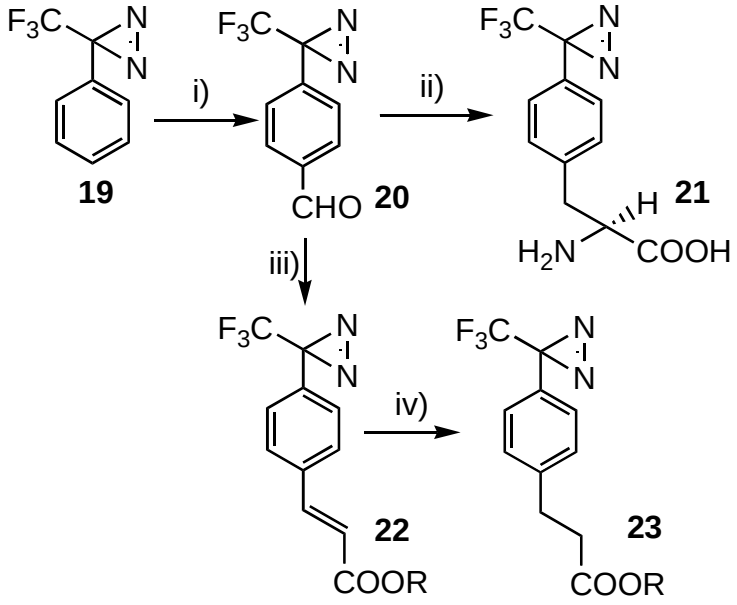


Figure 9

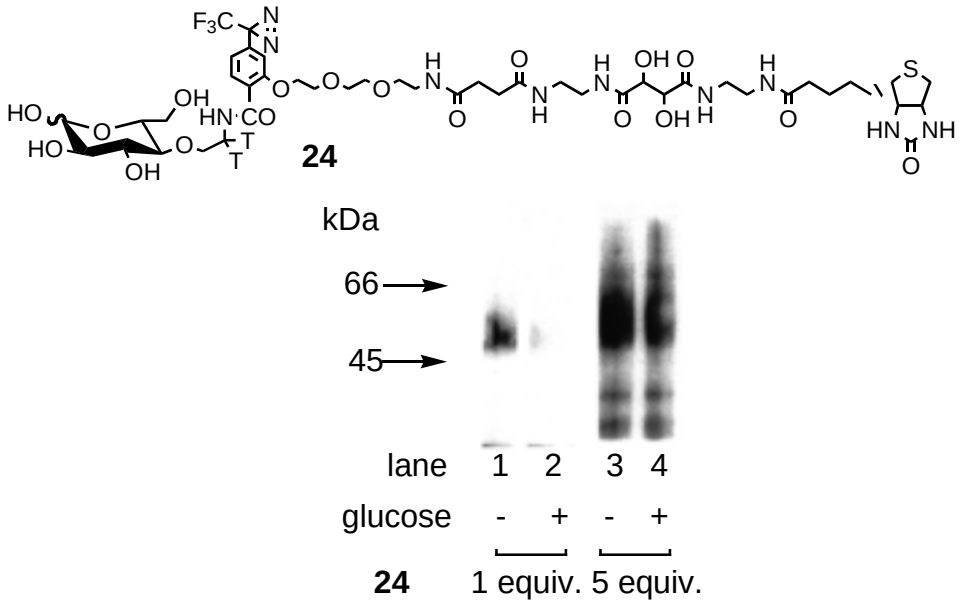


Figure 10

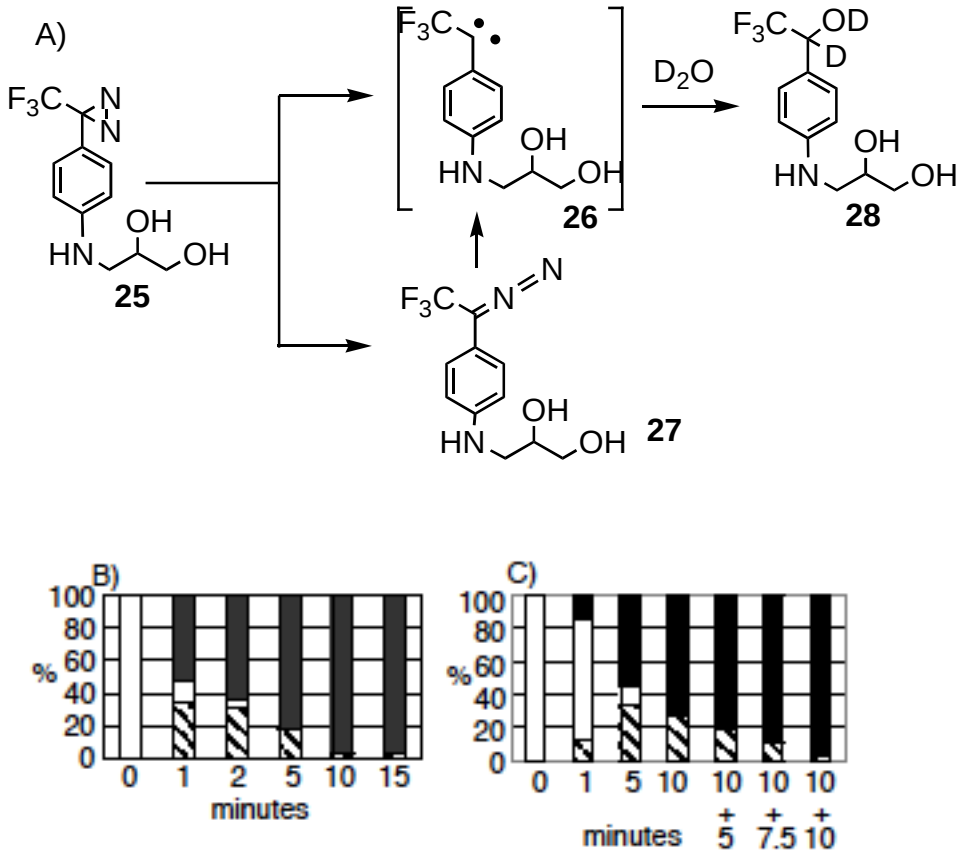


Figure 11

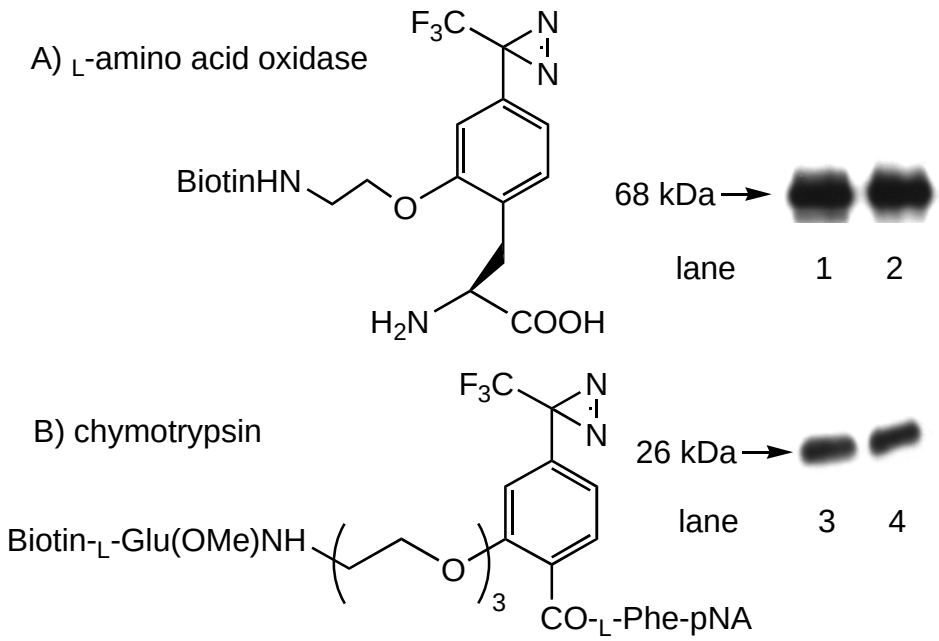
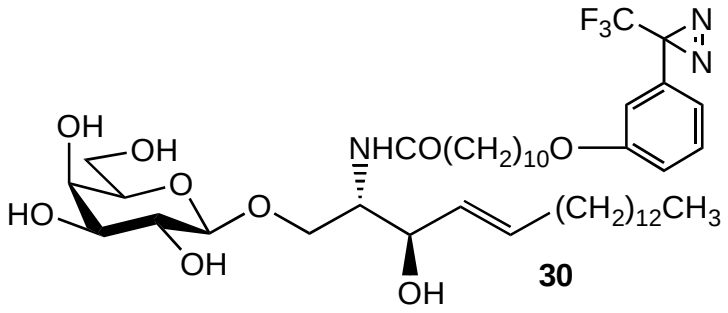


Figure 12



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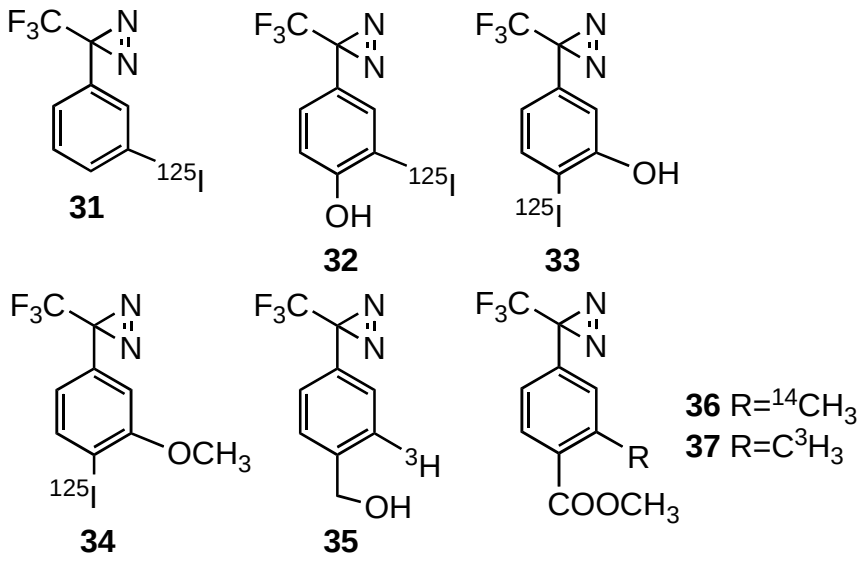

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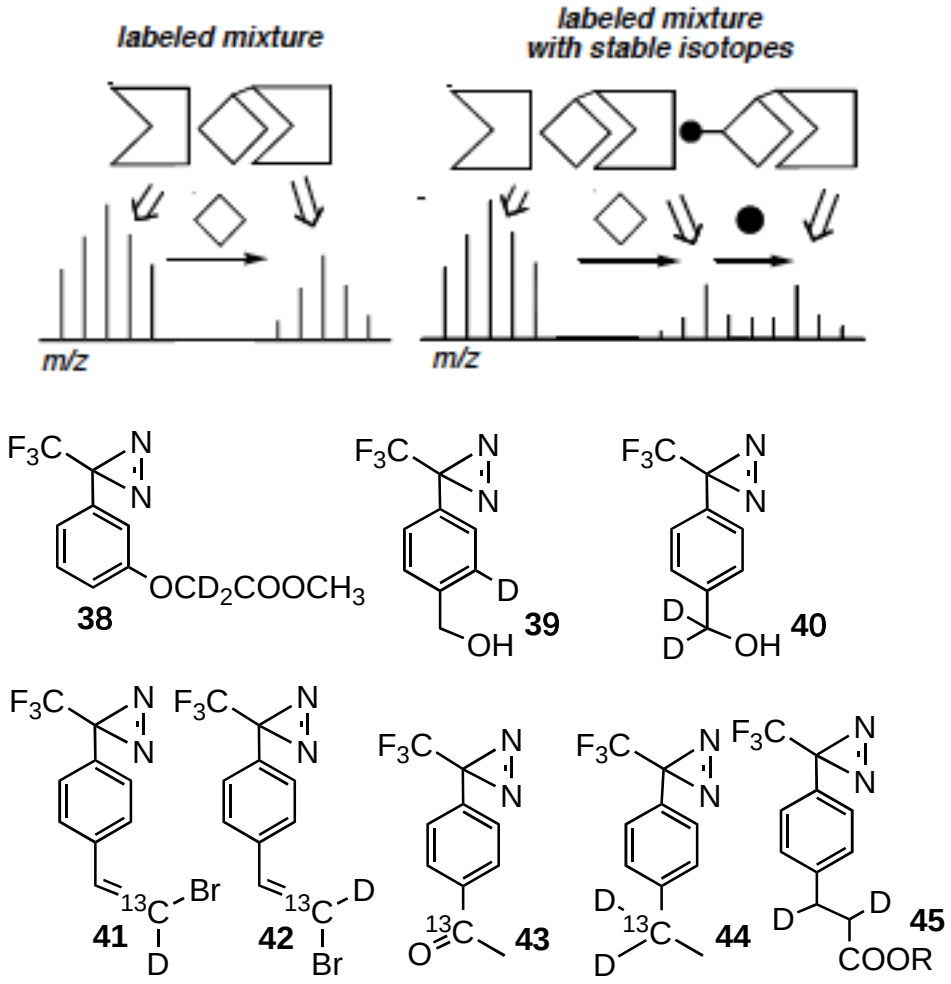


Figure 15

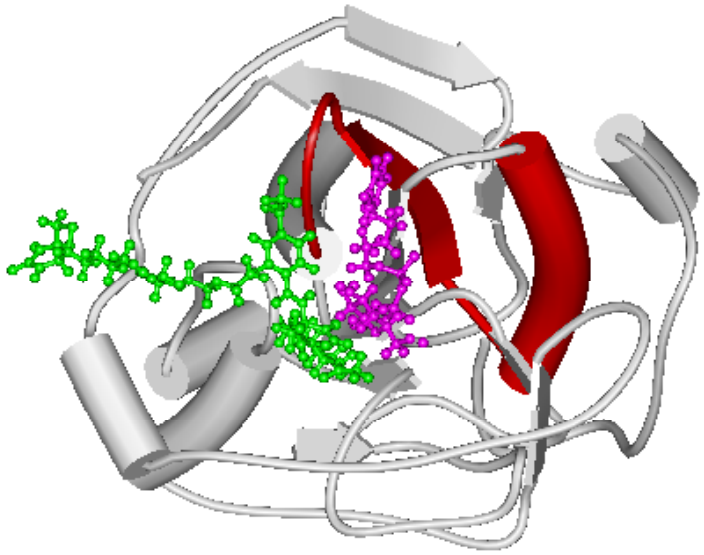


Figure 16

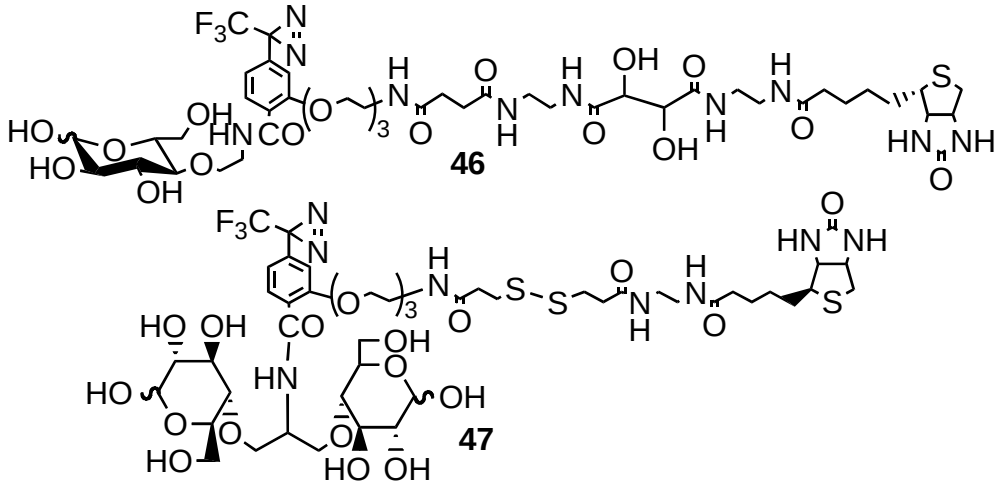
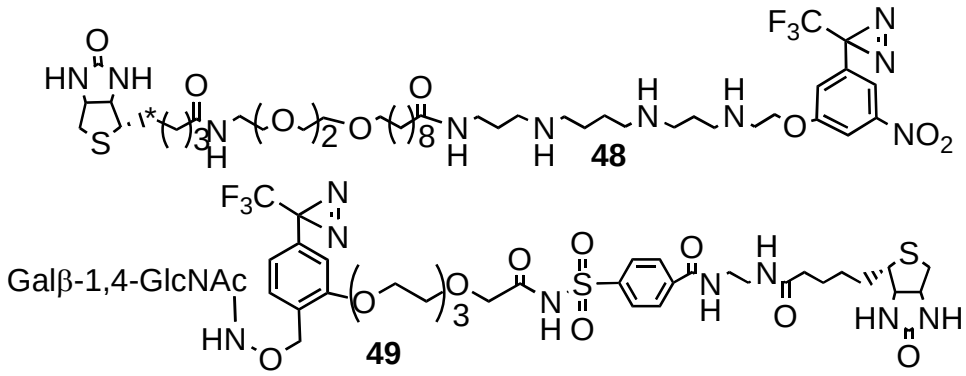


Figure 17



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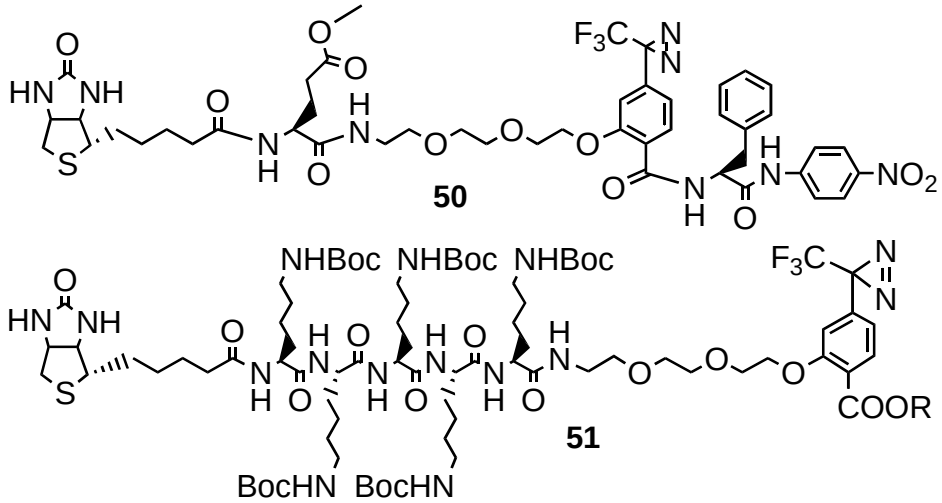

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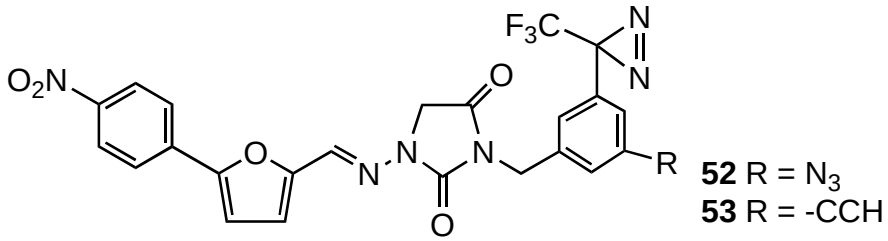

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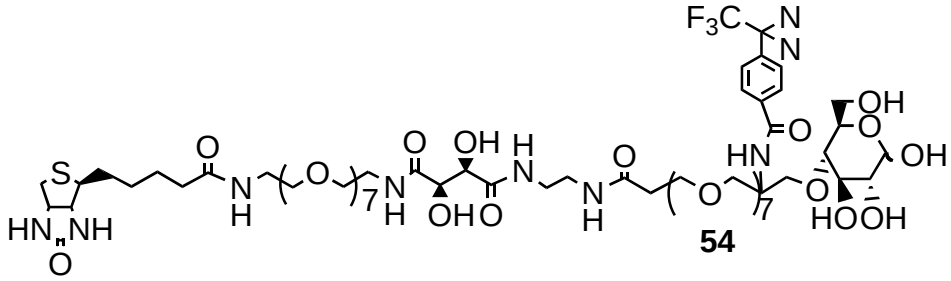

Graphical abstract