BIOTINYL DIAZIRINE PHOTOPHORE: AN APPROACH TO HIGH-RESOLUTION PHOTOAFFINITY LABELING FOR PROBING RECEPTOR-LIGAND INTERFACE

Yasumaru Hatanaka*[a] and Yuichi Kanaoka[b]

[a]Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan [b]Toyama Women's College, 444 Gankaiji, Toyama 930-01, Japan

Abstract - The technique of photoaffinity labeling has become increasingly appreciated as a powerful chemical methodology for the detailed structural analysis of ligand binding domains. This review describes our recent approach to the development of new phenyldiazirine family, the design of biotinyl photoprobes, and their application in the field of glycobiology.

1. Approaches to structural biology

Based on the recent development of recombinant techniques, the investigation of biofunctional machinery at their ligand accepting interfaces has become a challenging and important subject of structural biology in the oncoming new century. Three major approaches currently used in the investigation of receptor functional sites have their own advantages and limitations (Figure 1).[1] Spectroscopic methods are useful for analyzing ligand-receptor interactions at the atomic level.[2] These approaches, however, usually require a significant amount of stable and pure proteins. Protein engineering based on gene technologies provides a series of mutants for the structural analysis of functional sites. One prerequisite for the use of these methods is that the mutants must, to a large extent, retain the conformation of the native receptors.

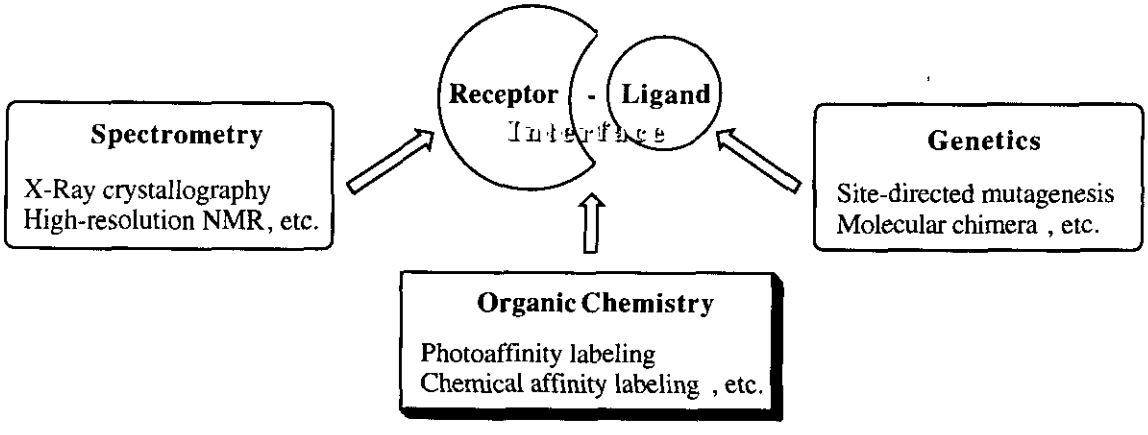


Figure 1. Approaches to the molecular recognition mechanism of receptor-ligand interactions.

However, it is generally impossible to exclude potential conformational changes which may result in the alterations of ligand binding processes. Photoaffinity labeling, one of the third independent approach, is a reliable chemical method which should be considered as being complementary to, rather than in competition with the other two approaches.[3]

2. New family of phenyldiazirine

Photoaffinity labeling is predominantly used for the identification of target receptors from a very crude protein mixture (first step in Figure 2). However, there are only few reports of identifying the detailed labeled regions within ligand binding sites (second step in Figure 2). The identification of retinal binding domain within rhodopsin protein by Nakanishi *et al.* could be a recent successful example of this category.[4]

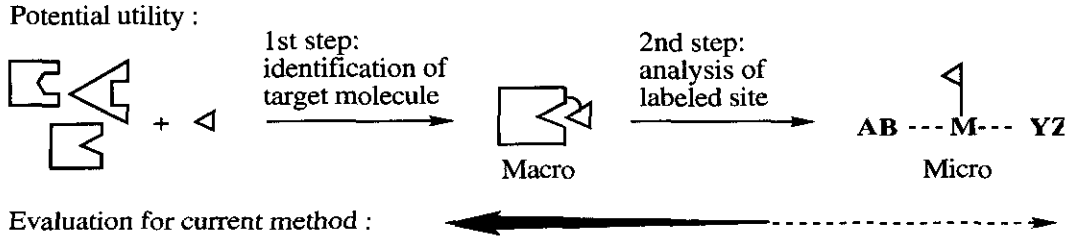
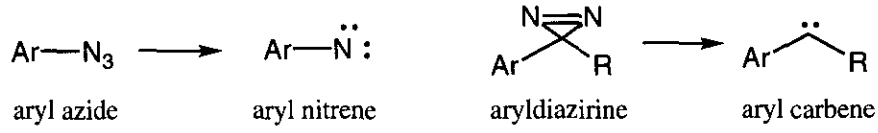


Figure 2. Schematic diagram of photoaffinity labeling.

Nitrene-generating aryl azides and carbene-yielding aryldiazirines are typical photoreactive groups currently used in photoaffinity labeling (Scheme 1).[5] Although carbenes are considered to be more reactive than nitrenes,[6] the ease of synthesis of aryl azides has significantly contributed to their widespread use for the identification of various receptors. Cross-linking with carbenes involves the formation of stable carbon-

Scheme 1



based bonds, whereas that with nitrenes formally produces nitrogen-carbon bonds or labile nitrogen-heteroatom bonds. Indeed, the covalent bonds formed with aryl azides were reported to be unstable under the typical experimental conditions of photoaffinity labeling.[7] Aryldiazirines were initially described by Knowles *et al.* for the displacement to the azide.[8] The search in this series led to the discovery of 3-aryl-3-trifluoromethyldiazirines by Brunner *et al.*[9] Although this diazirine appear to come closest to satisfying the criteria required for photoprobes, many steps are needed for the construction of the three-membered heterocyclic ring.[3] We, therefore, first established a practical method for the synthesis of this particular diazirine at scales of more than 0.1 mole.[10] By starting from some simple diazirines which can be synthesized on a large scale, the time-consuming diazirine synthesis was markedly simplified.[11] The first versatile approach involving direct substitution on the aromatic ring of phenyldiazirine was established by means of the aromatic thallation.[11] This new approach led to the

development of a family of phenyldiazirines without repeating all the steps of diazirine synthesis from the beginning (Figure 3). Very recently, the poisonous thallium reagent used in the key step of derivatization has been successfully replaced by less harmful $TiCl_4$. [12] This method enabled the introduction of aldehyde which is an easily modifiable key functional group for the further elaboration of this photophore.[12]

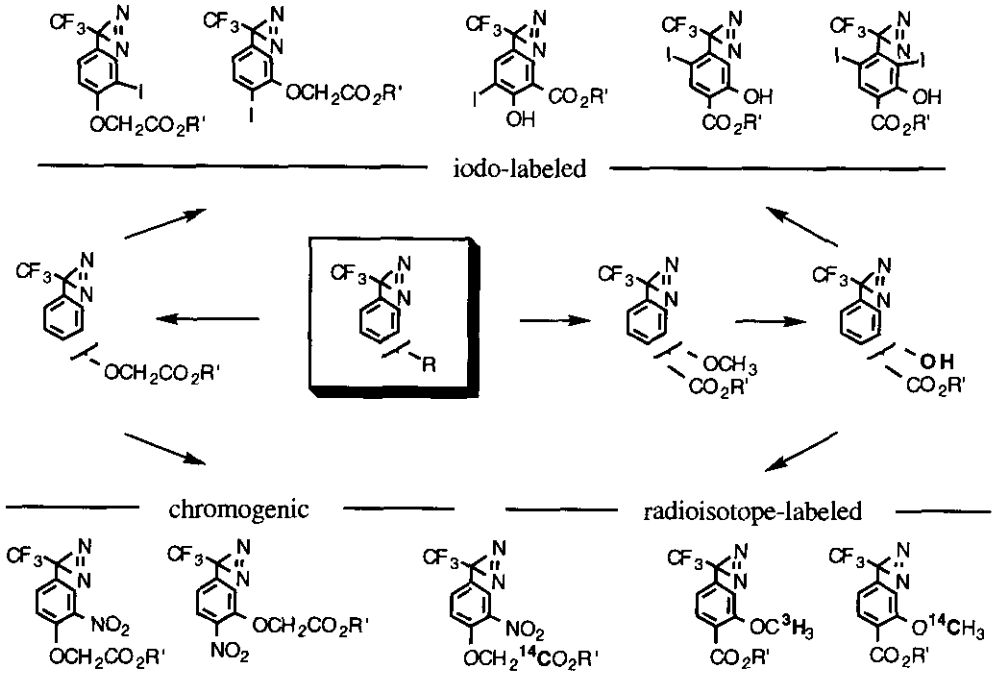


Figure 3. Novel family of phenyldiazirines for photoaffinity labeling.

3. Limitation of conventional photoaffinity labeling

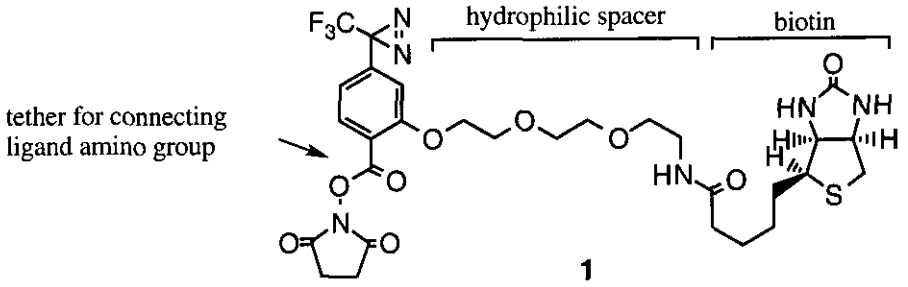
Due to the quenching of photochemically generated carbene or nitrene by co-existing water and buffer components, the yields of cross-linking produced by these reactive intermediates are usually very low. Our photoaffinity labeling study of ion channels revealed that labeling with diazirinyl probes was more efficient and stable compared to that with azide probe.[13] The yield of incorporation, however, was still low even with the diazirinyl probes, and a large amount of unlabeled protein remained in the reaction mixture. The low content of labeled channel proteins prevented us isolating the labeled peptide from a huge number of unlabeled fragments by high-performance liquid chromatography of digest mixtures. Application of a series of sequence-specific antibodies to this complex system eventually led us to the identification of labeled regions within the sodium channel protein as well as the calcium channel protein.[14]

4. Photochemical biotinylation

4. 1. Novel biotinyl diazirine. Many tasks including peptide synthesis are, however, required for obtaining a set of sequence-specific antibodies for the analysis of labeled regions.[15] Biotin strongly binds to avidin and a wide variety of avidin-biotin technology has been frequently used in various areas of biological research.[16] For the microscale handling of photolabeled products, the use of biotinylated

photoprobes could be an alternative method to the antibody techniques. The recent development of several biotinylated probes based on the azide photochemistry may be noteworthy in this regard.[17] Taking advantage of the easy functionalization of the diazirine family, we have now synthesized the first example of biotinylated diazirine **1** (Scheme 2).[18]

Scheme 2



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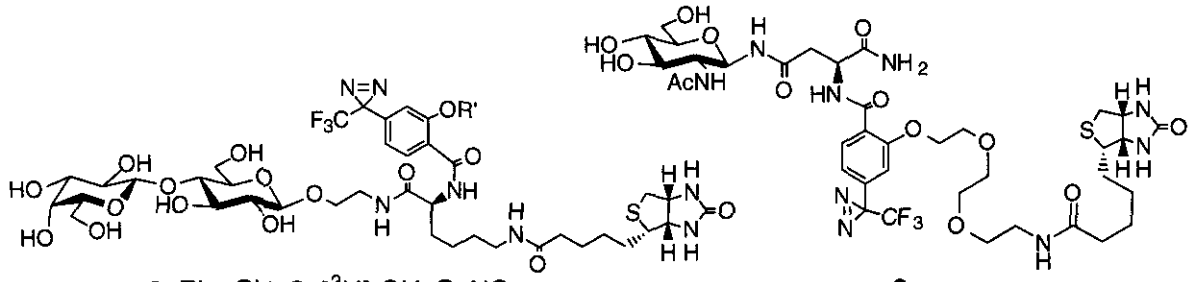
4. 2. Biotinylated photoreactive carbohydrates for the study of glycobiology. In recent years, the field of glycobiology has become increasingly appreciated when scientists working in the frontiers of life science have begun to discuss biological significance of carbohydrates.[19] Glycosyltransferases catalyze biosynthesis of oligosaccharides and the molecular basis for substrate recognition of these enzyme is the current topic in glycobiology.[20] We synthesized several biotinylated carbohydrates bearing phenyldiazirines for photoaffinity biotinylation of glycosyltransferases (Scheme 3). The biotinylated lactosyl ceramide analogs (**2**) were prepared as useful photoprobes for GM_3 synthase which is a key sialyltransferase in the biolosynthesis of gangliosides.[21] Asparagine-linked *N*-acetylglucosamine derivative (**3**) was also successfully applied to the photoaffinity labeling of β-1,4-galactosyltransferase.[22]

Scheme 3



2: R' = CH_3O, [^{3}H]-CH_3O, NO_2 **3**

4. 3. Photoaffinity biotinylation of β-1,4-galactosyltransferase

4. 3. 1. Chemiluminescent detection. β-1,4-Galactsyltransferase (GalT) catalyzes the transfer of galactose from UDP-galactose to the terminal *N*-acetylglucosamine or glucose to form β-1,4-glycosyl bond. The enzyme GalT is one of the most extensively studied glycosyltransferases, and was first cloned and sequenced.[20] The enzyme GalT is proposed to consist of the common domain structure of glycosyltransferase family whereas few regions of homology have been found within the catalytic domain of GalT. So far, the next step to interpret the region of substrate binding sites based on the search of sequence

homology has been unsuccessful. To evaluate the suitability of this biotinylated photoprobe, we have applied **3** for attaching the biotin tag within the acceptor substrate binding site of GalT.[22] Among the various techniques of avidin-biotin complex, we examined a chemiluminescent detection method for highly sensitive visualization of labeled components and an immobilized avidin matrix for the selective retrieval of labeled products. The electropheresed and electroblotted bands of photolabeled GalT were easily visualized by chemiluminescent detection using a streptavidin—horseradish peroxidase (HRP) conjugate catalyzed signal amplification method.[23] The result clearly showed the specific incorporation of the biotin tag into the bands corresponding two forms of GalT proteins (Figure 4).

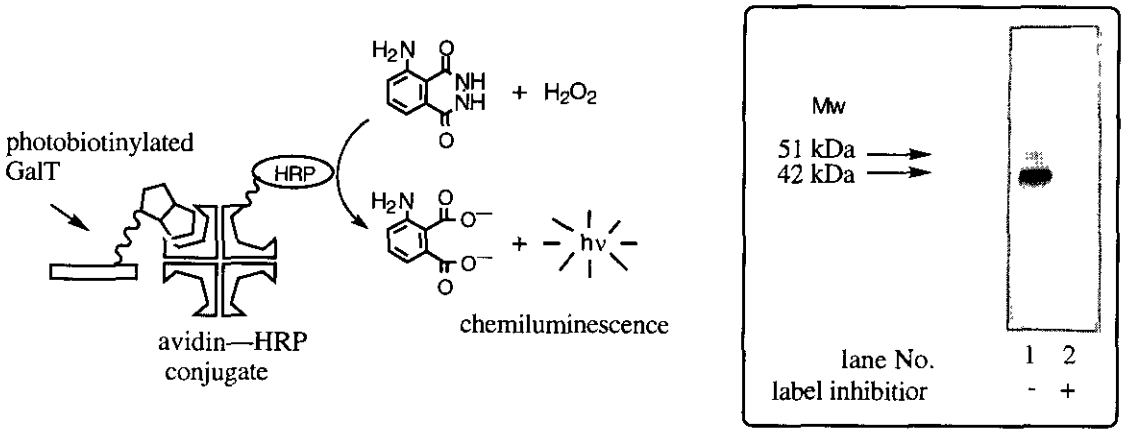


Figure 4. Chemiluminescent detection of photoaffinity biotinylated bovine GalT.
Photolabeled GalT proteins incubated at 37 ℃ in the absence (lane 1) or presence (lane 2) of label inhibitor.

4. 3. 2. Selective retrieval of photolabeled protein. After an immobilized streptavidin treatment (Figure 5, left), most of protein was recovered in the fraction passed through the column (Figure 5, right, panel A,

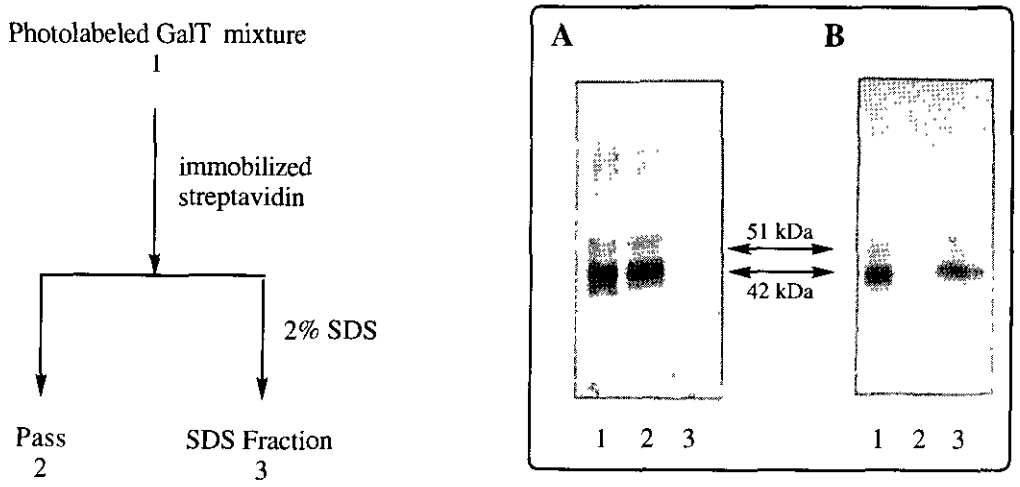


Figure 5. Affinity purification of photolabeled GalT.
Left: Procedures for chromatography of photolyzed bovine GalT on an immobilized streptavidin.
Right: (A) Silver-stained polyacrylamide gel; (B) Photographic result of chemiluminescent detection.

lane 2) and no detectable amount of GalT proteins was contained in the SDS fraction (A, lane 3). In contrast to this, no detectable chemiluminescence was observed in the pass-through fraction (panel B, lane 2) and the photobiotinylated GalT proteins were selectively retrieved in the SDS fraction, B, lane 3).

4. 3. 3. Probing the possible dynamic processes of GalT protein. The yield of specific labeling, the labelings that can be competed out with label inhibitors, was found to be dependent on incubation conditions of GalT proteins prior to the photolabeling. A progressive decrement in the yield of specific photolabeling was observed when the incubation temperature was successively lowered from 37 ℃ to 20 ℃ or 4 ℃ (Table 1). The amount of photoincorporation was also decreased when UMP was not included in the incubation mixture. One of the possible explanations to our observation could be the presence of some conformational changes which are affected by UMP as well as the incubation temperature. Before the binding of substrates, the catalytic domain of GalT may be rather exposed so as to facilitate the inclusion of substrates (Figure 6, A). Acceptor substrates could bind weakly at this stage, and this may explain the low yields of specific labeling. The binding of both substrate, donor and acceptor, may induce an endothermic movement that brings binding domains closer together for facilitating the subsequent glycosyl bond formation (Figure 6, B). At this stage, the photoprobe would gain better binding to result in the observed temperature and UMP dependent increment of specific labeling. The processes of conformational changes may be promoted by binding of UMP and are disturbed by lowering the incubation temperature. Upon binding of the complete set of substrates, the two domains of GalT would endothermically move each other resulting in a more closed and rigid conformation of the structure and increasing the interactions between the two domains. These structural changes may have a crucial role to achieve highly specific nature of the glycosylation. Molecules structurally different from original substrates may bind to the recognition site but cannot induce the requisite conformational change to catalyze the normal enzyme reaction.

Temp.	UMP	Yield of specific labeling
37 °C	+	4.0 %
	-	1.7 %
20 °C	+	2.5 %
4 °C	+	0.6 %
	-	0 %

Table 1. Yield of labeled GalT.

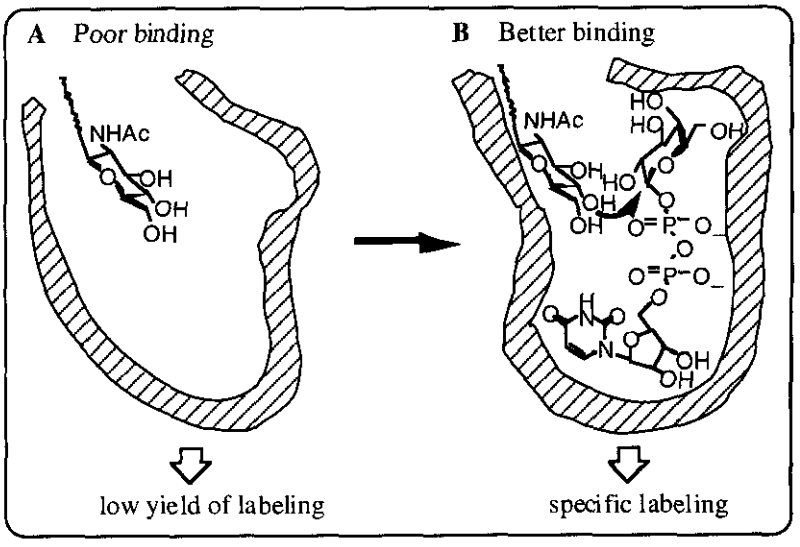


Figure 6 . A model for substrate-induced conformational change of GalT.

5. Conclusions

Since the discovery of 3-trifluoromethyl-3-aryldiazirines,[9] this class of compounds has been recognized to meet many of the criteria for an ideal reactive group of photolabeling reagents. Indeed, the diazirine probes were found to be superior to the classical azide probes for ion channel protein photoaffinity labeling.[13,14] Our versatile method for functionalization of phenyldiazirines developed a novel diazirine family without the need to repeat all the steps of diazirine synthesis from the beginning.[11,24] The ease of derivatization may lead to increasing use of diazirines as an alternative to aryl azides at the next stage of photoaffinity labeling. The covalent biotinylation using the novel diazirine-based photoaffinity probe described in this review seems useful for the radioisotope-free detection as well as the specific manipulation of photolabeled proteins. The method of affinity isolation described here has recently been applied for the isolation of photolabeled peptides from a extremely complex enzyme digest of GalT.[25] Because of the relative ease of experiments, the photoprobe carrying biotinyl diazirine will be a useful tool for further exploring the detailed molecular aspects of various biofunctional macromolecules. In combination with recent development of recombinant techniques as well as microsequencing methods, our biotinylated diazirine probes will be a promising answer for the high-resolution photoaffinity labeling. Photoaffinity labeling will continue to be an important method for elucidating structural aspects and molecular dynamics within cells, which may open up a new frontier, "cell organic chemistry," in the oncoming decade.

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