Synthesis of Diazirinyl Photoprobe Carrying a Novel Cleavable Biotin

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Photoaffinity labeling is a powerful tool for the identification of receptor proteins and their binding sites.[1] The major drawback of this method, however, is the reliability of the labeling and the purity of labeled peptides after purification from a large number of unlabeled fragments. The recent development of diazirine-based biotinylated probes provides an efficient solution to this problem. In these probes, the diazirine photophore contributes to the formation of a stable cross-link that greatly improves the efficiency.[2] The application of an *N*-acetylglucosamine photoprobe carrying biotinylated diazirine provided the first information regarding acceptor-site peptides of β-1,4-galactosyltransferase.[3] We have also developed a novel method for the one-step introduction of a biotinylated diazirine photophore into unprotected carbohydrate ligands.[4]

Since biotin–avidin binding is essentially irreversible ($K_d = 10^{-15}$ M),[5] several approaches have been investigated to achieve efficient recovery of biotinylated products from an immobilized avidin matrix. Although the use of monomeric avidin contributes to the isolation of biotinylated products because of the lower affinity to biotin ($K_d = 10^{-8}$ M), it requires a high

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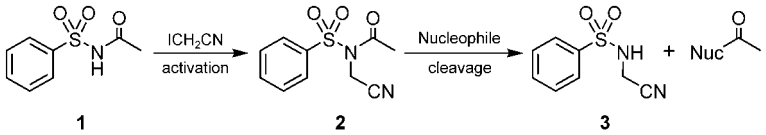


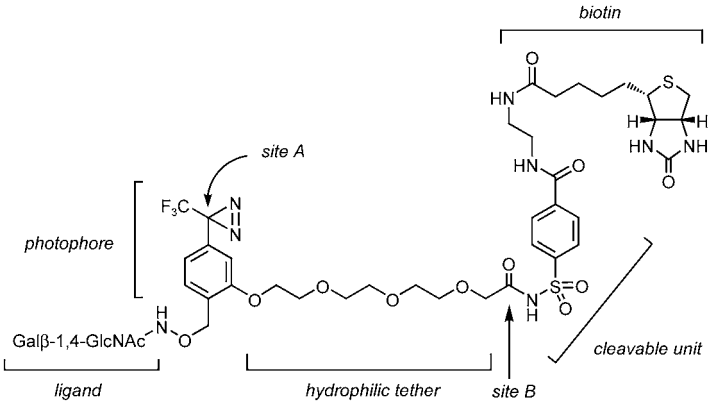
Scheme 1. *Cleavage procedure of acylsulfonamide.*

concentration of the biotinylated products for efficient trapping on the matrix.[6] Alternatively, biotinylated reagents with a scissile function have been considered for separation of the labeled proteins from a biotin–avidin complex. So as to displace labile disulfide groups,[7] photoreactive,[8] fluoride-sensitive,[9] alkali-sensitive,[10] and oxidative[11] linkages have been developed.

In this context, we applied acylsulfonamide chemistry to improve photoaffinity biotinylation. Acylsulfonamide is usually used for solid-phase peptide synthesis and combinatorial synthesis.[12] Although this group has never been used in protein cross-linking studies, it has attractive chemical characteristics compared to other cleavable cross-linkers.[13] 1) Acylsulfonamide is chemically very stable to strong acids and bases; this facilitates probe synthesis. 2) The NH proton of acylsulfonamide is acidic enough ($pK_a \approx 2$) to dissociate under physiological conditions; this might promote the key N-alkylation step for activating the desired cleavage. 3) Upon N-alkylation, the linkage is efficiently cleaved with nucleophiles under mild conditions (Scheme 1). Cyanomethylation was reported to be effective in the activation step,[14] whereas this step was optimized only in nonaqueous conditions. Biological application of the acylsulfonamide group has not been exploited. To avoid protein denaturation, we first examined the cleavage under weakly basic aqueous conditions using *N*-acetyl-*N*-(cyanomethyl)benzenesulfonamide (**2**) as a substrate. Although the yields of cleavage product under weakly basic conditions were rather low, the addition of ammonia significantly increased the yield. The yield of **3** in 0.1 M borate buffer at pH 9.0 with 0.02 M NH_3 was 60%, compared with a yield of only 30% without. The volatile nucleophile could be useful in the mass spectrometric analysis of proteins or peptides.

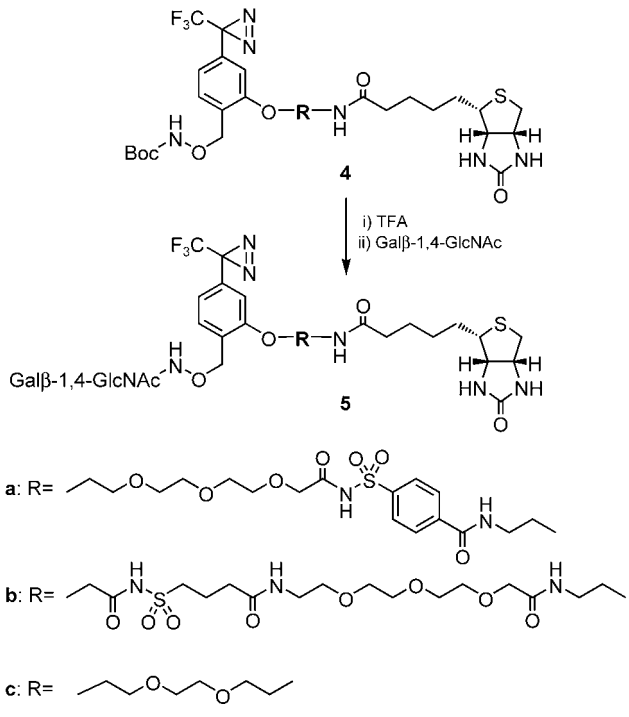
To apply the current approach, we investigated the affinity biotinylation of the galactose-specific lectin, *Ricinus communis* agglutinin (RCA) using Galβ-1,4-GlcNAc (LacNAc) as a ligand.[4] Scheme 2 shows the cleavage strategy of diazirine-based photoprobes. We prepared two types of biotinylated photoprobes, an aromatic sulfonamide **4a** and an aliphatic analogue **4b** (Scheme 3, for details see the Supporting Information). The latter linkage was reported to be cleaved more efficiently than an aromatic one in organic solutions,[13] whereas the aromatic linker seems to be preferable for the ease of synthesis of photoaffinity probes. A hydrophilic spacer unit was also introduced between diazirine and biotin. The Boc-protected aminooxy group of compounds **4a** and **4b** was selected as the connecting point for the one-step introduction of carbohydrate ligands.[4] After removal of the Boc group, LacNAc was easily coupled at pH 4 without protection to give the photoreactive probes **5a** (FAB-MS: $m/z = 1254$ $[M+H]^+$) and **5b** (FAB-MS: $m/z = 1277$ $[M+H]^+$) in yields of 53 and 45%, respectively.

The labeling ability and cleavability of the two cleavable probes **5a** and **5b** were compared with those of noncleavable probe **5c** by using RCA.[4] The photo-cross-linking reaction and detection of the products were performed by irradiation according to previous methods.[4] Figure 1 shows the 12% SDS-PAGE of photolabeled RCA proteins stained with Coomassie



site A: photolabeling
site B: can be cleaved by a nuclephile after alkylation of sulfonamide group

Scheme 2. *Design of a cleavable photo-cross-linker using acylsulfonamide.*



Scheme 3. *Preparation of cleavable and noncleavable photoprobes.*

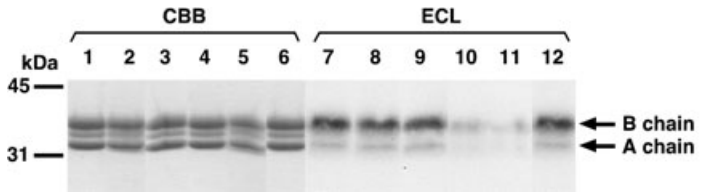


Figure 1. *Photoaffinity labeling of RCA was performed with LacNAc photoprobes* ***5a*** *(lanes 1, 4, 7, 10),* ***5b*** *(lanes 2, 5, 8, 11), and* ***5c*** *(lanes 3, 6, 9, 12). The products were detected by CBB-stained 12% SDS-PAGE (lanes 1–6) and chemiluminescent detection results of the products (lanes 7–12). Lanes 1–3 and 7–9 showed the results of the samples before cleavage of the sulfonamide linkages, and lanes 4–6 and 10–12 were the results of the samples after the cleavage.*

Brilliant Blue (CBB; lanes 1–6) and the chemiluminescent detection results (lanes 7–12) with an avidin–horseradish peroxidase (HRP) conjugate after blotting onto a poly(vinylidene fluoride) (PVDF) membrane (avidin–HRP/ECL, see Experimental Section for details). The RCA lectin is a 120 kDa tetramer protein consisting of two homologous subunits each composed of an A chain (32 kDa) and a B chain (37 kDa). Only the B chains possess an affinity for the terminal β-D-galactosyl residues, such as lactose, melibiose, LacNAc.[4,15] Lanes 1–6 showed that the amount of corresponding A and B chains was similar between the solutions before (lanes 1–3) and after (lanes 4–6) the cleavage reaction. A weak band between those of the A and B chains is known to be an impurity resulting from a closely related but distinct lectin.[16] The RCA proteins were stable, and no significant loss of total protein, for example by precipitation, was observed during the cleavage reaction.

Lanes 7–12 showed the labeled (biotinylated) products detected by avidin–HRP/ECL. When compared with the amount of subunits of original RCA shown in lanes 1–3, it was clear that all probes **5a**–**c** predominantly labeled the B chain (lanes 7–9). The efficiency of labeling was similar among the probes. A weak band due to the A chain was observed; however, it would have been labeled during the procedure after irradiation. Once the irradiated sample had been dialyzed to remove the unbound probes, the amount of signal was largely decreased. The irradiated samples were then treated with ICH_2CN followed by the addition of an ammonium solution in 0.1 M borate buffer at pH 9 (lanes 10–12). In the case of **5c**, which did not have a sulfonamide linkage, no significant change was observed between the products before and after treatment (lanes 9 and 12). In the cases of **5a** and **5b**, the amount of B chain was remarkably reduced after 1 hour's cleavage (lanes 10 and 11). The yields were approximately 76 and 78% for **5a** and **5b**, respectively. Consequently, both aromatic and aliphatic acylsulfonamide linkers were revealed to be useful for the analysis of biomolecules in aqueous solutions.

In addition, we verified the utility of these probes for RCA labeling. The amount of labeled products decreased with increasing amounts of lactose as a competitor,[15] and the bands disappeared in the presence of 0.1 M lactose (see the Supporting Information). These results indicated that these probes were preferentially recognized by the B chain of RCA.

Finally we investigated isolation of the labeled RCA from a streptavidin-immobilized matrix. Unbound probes were removed by gel filtration after photolabeling of RCA with the probes **5a**–**c**. Each of the irradiated samples was treated with mercaptoethanol to cleave the disulfide bond between the A and B chains. The solutions were then incubated with streptavidin-immobilized gel at room temperature. After the gel samples had been washed with 0.1% SDS-PBS to remove the adsorbed products, they were subjected to the cleavage procedure. After the reaction, the products in the supernatant were separated by SDS-PAGE. No signals were observed with a biotin-specific detection procedure (avidin–HRP/ECL, lane 2). Therefore, the supernatants reacted with biotin *N*-hydroxysuccinimide ester (NHS–biotin). The result for **5a** (lane 4 of Figure 2) shows that the chemiluminescence was predominantly detected in the zone corresponding to the band of the B chain at 37 kDa; compare the result in lane 1, which shows the original RCA proteins biotinylated by the reaction with NHS–biotin. In addition, conventional heat-denaturing was carried out instead

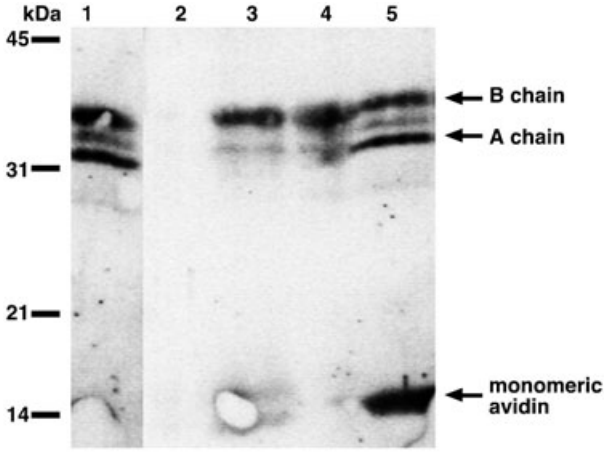


Figure 2. *Isolation of the* ***5a****-labeled RCA was carried out by using a streptavidin-immobilized gel. The samples in lanes 1, 4, and 5 were biotinylated by treatment with NHS–biotin before separation by 12% SDS-PAGE. The products were detected by avidin–HRP/ECL, lane 1: biotinylated original RCA proteins, lane 2: eluant from the gel after cleavage, lane 3: eluant from the gel after heating at 95°C for 15 min in PBS containing 2% SDS and 0.4 M urea, lane 4: biotinylated samples of the products in lane 2, lane 5: biotinylated samples of the products in lane 3.*

of the cleavage reaction by using ICH_2CN, and the products were detected by avidin–HRP/ECL before (lane 3) and after (lane 5) the treatment with NHS–biotin. The results indicated that nonspecific binding proteins on the gel, including unlabeled RCA proteins, could be eluted by heat denaturation, in addition to the labeled B chain (lane 3). These proteins could not be detected after the cleavage reaction (lanes 2 and 4). These results suggested that the bands in lane 4 are mainly from the photolabeled RCA that was eluted from the gel by cleavage of the acylsulfonamide linkage. Nonspecific binding proteins adsorbed on the matrix sometimes gave serious problems for purification of the target. In this case, the amount of nonspecific adsorption was not negligible compared with that of specific binding proteins. Under severe elution condition,

such as heat denaturation, even the proteins strongly adsorbed on the gel could be dissociated. Furthermore, the heat-denaturation method also yields the avidin subunit (14 kDa, lane 5) released by denaturation from the streptavidin-immobilized gel. Although the disulfide group has often been used as a popular cleavable linker, denatured monomeric avidin is also a contaminant. A combination of streptavidin–Sepharose and thiopropyl–Sepharose affinity chromatography was required for further purification.[17]

In conclusion, a new photolabeling technique based on an acylsulfonamide group was combined with a cleavable photophore to purify biotin-labeled proteins. This procedure gives great advantages for recovery of the targets trapped on the streptavidin matrix in aqueous as well as organic solutions. One of the major advantages of this method is that labeled proteins with high purity could be obtained under simple and mild elution conditions without significant levels of contamination. Recently, an enzymatic method for the cleavage and recovery of labeled proteins under mild conditions by using V8 protease was reported in which a glutamyl residue was introduced into the linker moiety.[18] However, this group may have inherent limitations due to the extensive enzymatic digestion required for labeled-site determination.

Finally, all of the procedures from affinity labeling to isolation can be carried out within a few days. This approach may extend the potential of photoaffinity labeling to become a more sensitive means for the rapid elucidation of protein structures and binding sites.

Experimental Section

General: The biotinylated diazirines **4a**–**c** were derived from 3-(3-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine (see the Supporting Information). RCA was purchased from the Honen Corporation (Tokyo, Japan). LacNAc were obtained from the Seikagaku Corporation (Tokyo, Japan). Streptavidin-immobilized gel (UltraLink Immobilized Streptavidin Gel) was purchased from Pierce Biotechnology (Rockford, USA). Anti-biotin HRP was obtained from BioLabs (New England, USA), and ECL Western blotting detection reagent (ImmunoStar) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Kieselgel 60 (70–230 mesh, Merck) was used for column chromatography. All chemicals were of analytical grade and were used without further purification. The reaction flasks containing diazirine derivatives were protected from light by wrapping with aluminum foil. The fast atom bombardment (FAB) mass spectra were obtained by using 3-nitrobenzyl alcohol as a matrix.

Preparation of LacNAc photoprobe: The typical procedure was as follows. Compound **4b** (19.8 mg, 20 μmol) was treated with TFA-CH_2Cl_2 (0.4 mL) at 0 °C for 30 min. After removal of the solvent, the residue was dissolved in 80% aqueous acetonitrile (0.5 mL) containing LacNAc (3.8 mg, 10 μmol). The solution was adjusted pH to 5–6 with diisopropylethylamine, and was incubated at 37 °C for 40 h in the dark. Separation by HPLC column chromatography on silica gel (Aquasil SS-1251, Sensyu Kagaku, Japan) gave the isomeric mixture of the glycoconjugates. Electronic absorption of the product was monitored at 360 nm.

Photo-cross-linking of RCA: Irradiation and analysis were performed by following established procedures.[4] RCA (10 μM) was incubated with each probe **5a**–**c** (10 μM) in phosphate buffer (0.1 M, 0.1 mL, pH 7.6) at room temperature for 30 min in the dark. The solutions were then irradiated at 0 °C for 40 min with a 30 W long-wavelength UV lamp (Funakoshi XX-15). The inhibition assay was performed in the presence of lactose (0.1 M) as inhibitor.

Chemiluminescent detection of photolabeled products: After irradiation, dialysis of the samples was carried out in PBS at 4 °C overnight. Each of the samples was treated with 10% mercaptoethanol solution containing 4% SDS. Each of the samples was separated by 12% SDS-PAGE, and protein bands were electrotransferred onto a PVDF membrane (Immobilon P, Millipore) in glycine (192 mM), Tris-HCl (25 mM), 20% methanol, and 0.1% SDS. The membrane was blocked for 1 h at room temperature with PBS containing 0.1% Tween and 2% skimmed milk. After the membrane had been soaked in avidin–HRP conjugate for 1 h at room temperature, it was treated with a chemiluminescence-detection reagent, and the emission was detected by using an imaging system (ChemiDoc, Bio-Rad Laboratories). This detection procedure was abbreviated as "avidin-HRP/ECL".

Cleavage of sulfonamide linkage of labeled RCA: The irradiated samples were treated with ICH_2CN (10 mM) in borate buffer (0.1 M) at pH 9 and room temperature for 1 h, followed by the addition of NH_4OH (20 mM) The mixture was incubated for 1 h.

Isolation of labeled RCA by using streptavidin-immobilized gel: After irradiation, unbound probes were removed by gel filtration (Sephadex G-50). Each sample was treated with 10% mercaptoethanol solution containing 4% SDS, and then incubated with streptavidin-immobilized gel (0.5 mL) at room temperature for 30 min. Gel samples were washed with 0.1% SDS-PBS and then subjected to the cleavage reaction. Instead of the cleavage reaction, another gel sample was suspended in PBS containing 2% SDS and urea (0.4 M) and then heated at 95 °C for 15 min. After these reactions, all of solutions were briefly separated in a centrifuge, and the supernatants were treated with NHS–biotin (2 μL of 0.1 M DMSO) at room temperature for 1 h. Each sample was analyzed by 12% SDS-PAGE and detected by avidin-HRP/ECL as described above.

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Keywords: cleavable linkage · cross-linking · diazo compounds · glycoconjugates · photoaffinity labeling

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